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Radioactive Iodinated Human Serum Albumin as Tracer Agent for Diagnosing and Localizing Intracranial Lesions.* (18720)

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(Introduced by R. L. Varco.)

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Various isotopes have been used to diagnose and localize intracranial lesions by the isotope-encephalometric technic described by Moore (2-4). So far, diiodofluorescein has given the most satisfactory results and therefore gained the widest acceptance. However, at this clinic radioactive-iodinated-human-serum albumin has been used and appears to be as satisfactory, if not superior, to diiodofluorescein(1). The iodination of human serum albumin (RIHSA‡) is carried out with elemental iodine in an essentially neutral buffer. One cc of RIHSA contains 5 mg of human serum albumin with a salt concentration of approximately 2 mg(6). Metabolic studies in both animals and humans have shown that RIHSA given intravenously remains in the blood stream for a long period of time. It is apparently gradually metabolised by the body and free iodine liberated. Although most of the radioactive iodine is excreted through the urine, about 5% of it is taken up by the thyroid gland by the end of 24 hours(5). A small amount of RIHSA, which is not readily metabolized, diffuses out into the tissue and into lymphatics unchanged. About 1-2% of the tagged albumin can be obtained from the thoracic duct by the end of one hour. The liver, however, apparently does not participate in the excretion of RIHSA. It has been shown in experimental animals that only a negligible amount of RIHSA can be recovered in the bile(5). Kidneys are the main organs that are responsible for the excretion of I^{131}. At the end of the first day, the total urinary excretion reaches about 15% to 20% of the total amount given. The blood as well as the tissue level gradually falls and eventually all the injected RIHSA is metabolized and I^{131} excreted by the kidneys.

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* Supported in part by the U. S. Atomic Energy Commission and the U. S. Public Health Service.

† On active duty. M. C., U. S. Army.

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‡ Obtained from the Abbott Laboratories, North Chicago, Ill.

The uptake of I^{131} by the thyroid raises the question of whether or not it is safe to use RIHSA as a tracer agent to diagnose intracranial lesions. However, it is possible to prevent the deposition of I^{131} in the thyroid. It has been shown that stable iodine administered to patients as Lugol's solution for a week starting at least 24 hours previous to the administration of RIHSA will block the uptake of I^{131} by the thyroid and thereby eliminate the danger of excess irradiation.

At this clinic, the present regime requires intravenous administration of from 400 to 500 μc of RIHSA to the patient who has been given 10 drops of Lugol's solution, 3 times daily at least 24 hours prior to the test. An interval of not less than 30 minutes should elapse before the head is monitored. However, when time is available the initial counting should be done 24 hours later in order to get a more significant differential "uptake" between the tumor and normal brain. It is important, for the purpose of obtaining counts during the optimal time when ratio between the tissue concentration and blood concentration is the greatest, that the patient is counted daily for 2 to 3 days. A focus of high uptake obtained by the initial counting after injection should persist through the second and third or even fourth days. In fact, our experience has been that the differential uptake between the site of the lesion and the symmetrical contralateral area, should increase on the second and third day. Table I illustrates this point. Ten cases have been subjected to this technic up to date. We were able to localize tumors in 5 cases, subdural hematoma in one case, and rule out tumors in 2 cases which subsequently had both negative air studies and angiograms. The remaining two patients were still undergoing study.

TABLE I. D.M. ♂ 25 U.H. #829584. 460 μc RIHSA Given I.V.

	c/m	%D
First survey—1 hr after administration		
R_3	1857	L
L_3 Lateral frontal	2005	7%
L_5	2823	L
R_5 Midtemporal	2532	10%
R_6	1641	L
L_6 Temporo-parietal	1686	2.5%
L_7 Parasagittal	1798	0
R_7 (earline)	1801	0
R_9	1656	L
L_9 Post-temporal	1712	3%
L_{10}	1867	L
R_{10} Post-parietal	1843	1%
Second survey—24 hr later		
R_3	1978	L
L_3 Lateral frontal	2163	8%
L_5	2154	L*
R_5 Midtemporal	1934	10%
R_6	1593	L*
L_6 Temporo-parietal	1931	17%
L_7 Parasagittal	1974	L*
R_7 (earline)	1749	11%
R_9	1499	L*
L_9 Post-temporal	1796	16%
L_{10}	1872	L
R_{10} Post-parietal	1811	3.5%

On the first day, only one position showed a differential uptake of 10%. On the second day, the positions over the tumor all showed a significant increase of differential uptake.

* Site of tumor—craniotomy—extensive left temporal astrocytoma.

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Admittedly, it is too early to predict what a role RIHSA will play in the diagnosis and localization of intracranial lesions. However, advantages are many: (a) It is much less expensive than diiodofluorescein (DIF*), (b) 400-500 μc will give about the same counting rate as 1 mc of DIF, (c) RIHSA stays in the tissue longer and therefore permits repeated counting, and (d) an increase in differential uptake by the focus on the 2nd and 3rd days survey will serve to confirm the presence of a lesion demonstrated in the initial counting.

The only disadvantage of this method is the uptake by the thyroid gland of I^{131} split off RIHSA. Although stable iodine serves to block this uptake, experience is too limited to say anything definite as to the amount of radiation the thyroid gland might receive as a result of RIHSA administration. However, with the adoption of scintillation type counters, it is possible that the counting rate will be increased to such an extent that the dosage of RIHSA can be greatly reduced to a much safer level.

It is our feeling that RIHSA and DIF may act in a more or less identical manner in the body. When DIF is administered, it may combine physically with the serum albumin of the patient. In other words, DIF is merely used as a reagent to tag the serum albumin of the patient. This hypothesis is being investigated.

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Diminished Adrenal Cortical Function in Diabetes as Shown in Eosinophil Response to Stress of Surgery. (18721)

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The relationship between the adrenal glands and the pancreas in diabetes mellitus has been the subject of investigation for many years. Recently several observations have been made which suggest, if anything, a reduction of adrenal cortical function in diabetes. Miller and Mason(1) measured the 17-ketosteroids in the urine of 64 adult diabetic patients and found that 88% of the values were below the average normal, while 27% fell below the minimal normal value. They were unable to correlate the excretion of 17-ketosteroids with either the age of the patient or the severity of the diabetes. No mention was made of a possible relationship to the duration of the disease. These investigators concluded that diabetes often has an effect on the adrenal glands which results in a lowered excretion of 17-ketosteroids which is not related to the degree of impairment of carbohydrate metabolism. This finding of reduced 17-ketosteroid excretion in diabetes has been reported by several other workers(2-4). Forbes *et al.*(3) postulated that the decrease was due to the accompanying secondary malnutrition rather than the diabetes *per se*.

The 11-17 oxysteroid excretion in diabetes has also been measured(5,6). Although none of the series is large enough to be statistically significant, the results seem to confirm the trend toward lowered adrenal cortical function in diabetes. The mean value for five diabetics as measured by Talbot was 0.058 mg/sq.m of body surface per day as compared to a mean value of 0.132 mg/sq.m of body surface per day for normals. Three of his 5 patients

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with diabetes had values below the lower limits of normal, while 3 of McArthur's 7 cases also had definitely diminished excretion of 11-17 oxysteroids. Wilson *et al.*(4) have also concluded from their experiments with pituitary adrenocorticotrophic hormone (ACTH) that the adrenal function in diabetes appears to be somewhat less than normal.

The present study was undertaken in an effort to learn more about the level of adrenal cortical function in diabetes mellitus by observing the eosinophil response of diabetics undergoing major surgery. In general the level of circulating eosinophils is closely related to adrenal cortical activity. The changes in circulating eosinophils following major surgical procedures have been reported by various investigators(7-9). Within 4 hours after operation there is a marked and sustained reduction in the eosinophil count, which is followed by an "eosinophilia" often greater than the pre-operative level. The initial eosinopenia may be construed as further evidence of the role of the adrenal cortex in the "alarm" reaction in man. Roche, Thorn, and Hills(9) have shown that in normal people undergoing major surgery the eosinophils are depressed at least 50% of the pre-operative level on the second post-operative day, approximately 48 hours after operation.

Methods and materials. Measurement of the eosinophil levels was done using venous blood by a modification of the method of Dunger as described by Hills *et al.*(10). The blood was collected in tubes containing balanced oxalate. If the counts were not done immediately, the blood was refrigerated. However, all counts were done within 24 hours after the blood was drawn. The ACTH (Armour) and 17-hydroxy-11-dehydrocorticosterone (Cortone, Merck & Co.) tests were performed using 25 mg of ACTH intramuscularly and 100 mg of Cortone orally. After doing an initial count, ACTH or Cortone was administered, and 4 hours later another eosinophil level was obtained. A fall in the eosinophils of 50% or more of the initial level indicates a normal adrenal cortical response and in all probability eliminates the possibility of adrenal cortical insufficiency(11). The Cortone was given mixed with 60 cc of orange juice. In normal people this amount is sufficient to reduce the number of circulating eosinophils by at least 50% in 4 hours(12). All of the tests were done in the morning, and meals were arranged so that the second level was obtained at least 3 hours after the last meal.

Results. The eosinophil level was determined on the second post-operative day in 25 diabetic and 13 non-diabetic patients undergoing major surgery. In each case a pre-operative count was done on the morning of operation. The average fasting circulating eosinophil level in the 25 diabetics was 247/cu mm, while in the 13 non-diabetics it was 287/cu mm. Ten of the 25 diabetics showed an eosinophil count on the second post-operative day which was depressed less than 50% of the pre-operative level. On the other hand, all of the 13 non-diabetic patients had eosinophil levels which were decreased more than 50% of the pre-operative count (Fig. 1). The difference between these two

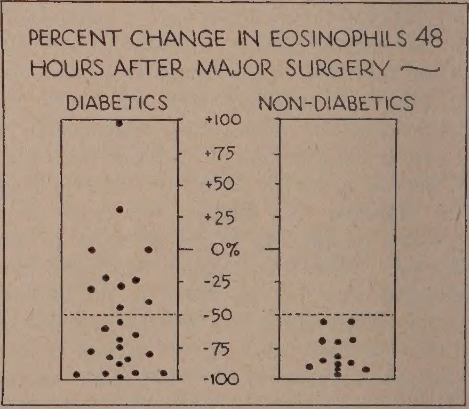

FIG. 1.

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TABLE I. Comparison of the Effects of Similar Operations in Diabetics and Non-diabetics.

	% change in eosinophils		
Operation	Diabetic with abnormal response	Diabetic with normal response	Non-diabetic
Hysterectomy	+28	–58	–70
Sympathectomy	+97		–57
Lobectomy	–25	–67	–89
Cholecystectomy	+ 1	–70	–73

groups is statistically significant. A deliberate attempt was made to include the same type of operation in both the diabetic and non-diabetic series. In the group of 10 diabetics who demonstrated diminished adrenal cortical function the following operations were represented: sympathectomy, hysterectomy, exploratory thoracotomy, exploratory laparotomy, lobectomy, thigh amputation, and cholecystectomy. Table I shows a comparison of the effects of similar operations on the eosinophil response in diabetic and non-diabetic patients.

Five diabetics who exhibited diminished eosinophil and thereby probably decreased adrenal cortical response to the stress of surgery were given 4-hour ACTH tests. In each case the fall in eosinophils was less than 50%, with a range of —2% to —39%. These tests were done during the third to seventh post-operative days when the number of eosinophils had again risen toward normal. This seemed to indicate some degree of adrenal cortical insufficiency inasmuch as non-diabetics exhibited a normal response to 25 mg of ACTH during the second to fifth post-operative days(9). However, the possibility still remained that the abnormal response in the diabetic was not due to adrenal inadequacy, but to unresponsiveness of the eosinophil itself. To demonstrate that the eosinophils were sensitive to adrenal cortical hormones, Cortone tests were performed in the 5 patients previously tested with ACTH. In every one the number of eosinophils dropped more than 50% in 4 hours. The range was from —51% to —85%. The results of the ACTH and Cortone tests in these five diabetics are shown in Fig. 2.

In order to determine whether the abnormal response exhibited by some diabetics represented an earlier occurrence of the normal third-day "eosinophilia" or a complete inability to react to the stress of surgery with any marked eosinopenia, the eosinophil levels of 8 patients were followed for several days post-operatively. In addition a count was obtained from 4 to 10 hours after operation. The results are illustrated in Fig. 3. In each case there was a marked reduction in eosinophils immediately following surgery. This eosinopenia continued until the second post-operative day when the eosinophil levels had risen to more than 50% of the pre-operative level in the patients with an abnormal re-

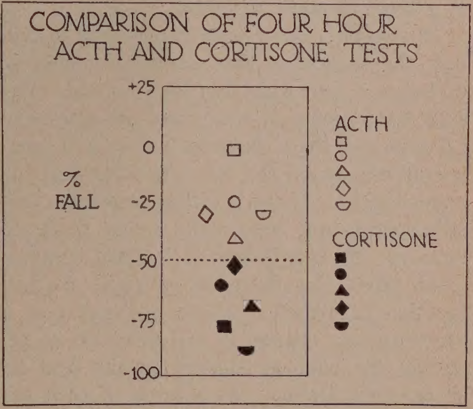

FIG. 2.

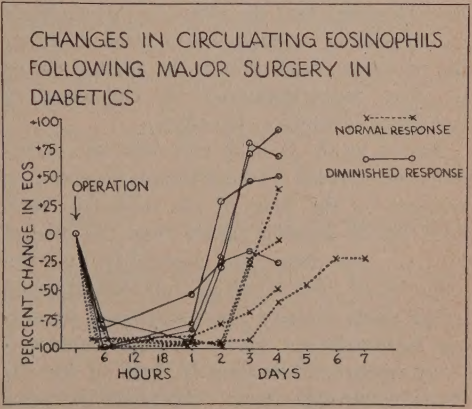

FIG. 3.

sponse. It was evident that the adrenal cortex was capable of responding normally to the initial stress of the operation, but that its reserve capacity was insufficient to maintain the eosinopenia for 48 hours.

The average age of all the diabetics who displayed an abnormal response was 50.6 years, and they had had diabetes for an average of 12.7 years, while the normally-responding diabetics averaged 57.1 years in age and had had their disease 8.4 years. Although the series is not large enough to be statistically significant in respect to this point, it suggests a correlation between the duration of the diabetes and the presence of diminished adrenal cortical response. There was no relationship between the severity of the diabetes as measured by the insulin requirement and the type of adrenal response to surgery.

Discussion. These observations tend to confirm the suggestion that adrenal function in diabetics is somewhat less than normal. Despite the presence of diminished adrenal cortical response, these diabetics did as well post-operatively as did the other diabetics and the non-diabetics. If one is willing to assume that adjustments are continually being made to maintain the body's normal internal environment, then the decreased adrenal cortical function in diabetes can be postulated as a compensatory change in the direction of restoring the balance between insulin and its antagonistic factors. As a result of their experiments on patients with Addison's disease and Cushing's syndrome, Wilson *et al.*(4) have proposed that the effect on carbohydrate metabolism of primary dysfunction of either the pancreatic islet cells or the adrenal cortex is often counterbalanced by compensatory functional changes in the other.

Forbes *et al.*(3) have explained the occurrence of lowered 17-ketosteroid excretion in diabetes on the basis of the secondary malnutrition of diabetes rather than the disease *per se*. This raises the possibility that the reduction of adrenal cortical function observed in this study is also a manifestation of secondary malnutrition. However, this seems unlikely when consideration is given to the patients in this experiment. All of the diabetics were patients of the George F. Baker Clinic and were hospitalized for several days prior to operation. During this period their diabetes, usually already under satisfactory control, was managed more strictly and their general nutrition improved. In this connection it is known that a high-protein diet increases adrenal cortical activity. Prior to hospitalization almost all of these diabetics were leading normal lives and were in average nutritional condition. Also against the contention that malnutrition played an important part in the reduced adrenal cortical function is the observation that three of the non-diabetic controls had tuberculosis and still exhibited a normal response to the stress of surgery. Three cases are scarcely enough to be significant, but they do suggest that poor nutrition is not necessarily the cause of the diminished adrenal cortical response seen in diabetes.

Summary. 1. The levels of circulating eosinophils as a measure of adrenal cortical response to the stress of surgery were followed in 25 diabetics and 13 non-diabetics. Ten of the 25 diabetics showed diminished adrenal cortical function as indicated by an eosinophil count on the second post-operative day which was depressed less than 50% of the pre-operative level. Four-hour ACTH and Cortone tests were done on 5 of these 10 diabetics and confirmed the presence of reduced adrenal cortical reserve. The adrenal cortex was capable of responding normally to the initial stress of surgery, but its reserve capacity was insufficient to maintain the eosinopenia for 48 hours. There is a suggestion that the abnormal adrenal response can be correlated with the duration of the diabetes. 2. The possibility is discussed that this decrease in the duration of the activity of the adrenal may be a compensatory change which results from the relative insulin deficiency of diabetes mellitus.

We wish to thank Dr. Peter H. Forsham for his helpful suggestions and criticisms and Drs. Herbert D. Adams, Bentley P. Colcock, and Richard H. Overholt for permission to use their patients as controls.

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Epinephrine Protection Against Fatal Hyperkalemia in Adrenalectomized Rats. (18722)

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Normal humans and animals tolerate a fairly large dose of glucose solution by mouth. It is also recognized, however, that a high carbohydrate intake may precipitate Addisonian patients into a crisis(1). The factors responsible for this difference in carbohydrate tolerance are not known. At this laboratory it was observed that the administration of 2 ml of a 50% glucose solution directly into the stomachs of adrenalectomized rats was followed shortly by death. Examination of the viscera of these animals revealed only that the hearts were greatly enlarged and that death had probably resulted from cardiac standstill. The association of elevated plasma potassium concentration with the rapid onset of cardiac standstill has been demonstrated (2). It is also known that an elevated plasma potassium level is unusually toxic in animals with adrenocortical insufficiency.

It is the purpose of this report to present evidence that epinephrine protected adrenalectomized rats against the mortal effect which followed the administration of a 50% glucose solution *per os*. Epinephrine was selected to be so tested since it was recently reported(3) that this substance significantly lowered the plasma potassium concentration of normal and adrenalectomized rats.

Materials and methods. Normal intact, bilaterally adrenalectomized and bilaterally adrenal enucleated groups of male Wistar rats weighing 225-250 g were used. All rats were given a chow biscuit *ad libitum* and greens twice weekly. The adrenalectomized and enucleated rats were maintained on 1% NaCl drinking solution. Adrenal enucleation was performed by the method of Ingle and Higgins(4). A solid core of medulla and cortex was extruded from a slit in the gland and the organ was then gently milked with a forceps. A histologically normal-appearing cortex is regenerated in a short time from residual cortical cells(5). Histological study* of the regenerated adrenal glands removed from these rats showed good regeneration of all 3 cortical zones and the absence of the medulla. These rats, therefore, are termed demedullated. The adrenalectomized and demedullated rats were used in the experiments 14-16 days after their surgery. After an overnight fast the rats were lightly anesthetized with n-methylcyclo-hexenyl-methyl barbituric acid (Evipal) immediately before the administration of 2 ml of either a 50% glucose solution (c.p. Dextrose, Merck), physiological saline or mineral oil (U.S.P., light) directly into the stomach by means of a fine plastic tube. A group of adrenalectomized and demedullated rats were pre-treated with epinephrine (adrenaline tablets, Parke, Davis & Co., prepared fresh by solution in physiological saline) injected subcutaneously at a dose level of 0.04 mg/100 g of body weight (approximately 0.5 ml) 60 minutes before the administration of the glucose solution. Another group of adrenalectomized rats was pretreated with 2 ml of lipo-adrenal extract (Upjohn)† injected subcutaneously 120 minutes before the administration of the glucose *per os*. This pretreatment time-period has been shown as adequate to make available to the rat a supply of exogenous adrenal steroids(6). After the

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* Acknowledgment is made to Dr. Leo D. Moss, Pathologist, Bradford Hospital, for the interpretations of the sections of the demedullated adrenal glands.

† The Lipo-adrenal Extract was generously supplied by Dr. H. F. Hailman of The Upjohn Co.

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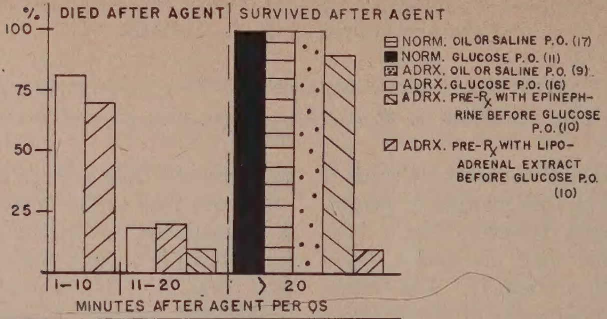

MEAN PLASMA LEVELS OF GROUPS ABOVE

K meq/l	7.9	6.5	8.6	6.2	6.3	4.2	4.1	4.4	4.3	4.8
±	.63	.1				.21	.08	.06	.13	
Glucose mg%	48	81		144	227	223	130	63	274	231

FIG. 1. Protection of adrenalectomized rats with epinephrine pretreatment against mortality and hyperkalemia following 2 ml of a 50% glucose solution *per os*. No. of rats in each group is in the parentheses. ± is the std error of the mean.

agent had been admitted into the stomach, each of the rats was observed carefully for the first signs of failure, respiratory difficulty or anoxia. At this time a cardiac puncture was made and blood withdrawn into a dry syringe containing 1 drop of heparin (Liqueamin, Lilly, 10 mg/ml). The blood was immediately centrifuged. Aliquots of plasma, if free from hemolysis, were taken for the determination of plasma potassium and glucose concentration. The potassium concentration (m.eq./L) was determined with a lithium internal standard flame photometer. Plasma glucose (mg%) was determined photometrically by the method of Kingsley and Reinhold(7). Since all the adrenalectomized rats showed signs of failure in less than 20 minutes after the glucose solution had been admitted into the stomach, those rats which had no signs of failure for 20 minutes were accepted as probably surviving the procedure and sacrificed 25-30 minutes after the agent had been given.

Results. Adrenalectomized groups. Inspection of Fig. 1 shows that 100% of a group of adrenalectomized rats were mortally affected less than 20 minutes after 2 ml of a 50% glucose solution was admitted directly into their stomachs. Pretreatment of another group of adrenalectomized rats with 2 ml of lipo-adrenal extract had not altered the results. However, pretreatment with a single injection of epinephrine 60 minutes before giving the glucose solution *per os* protected another group of adrenalectomized rats. It is apparent from the results obtained in the control groups (the normal rats given glucose solution, and the normal and adrenalectomized groups given either physiological saline or mineral oil *per os* that the procedure, *per se,* was innocuous. Inspection of the plasma potassium concentration of the groups in Fig. 1 shows that the potassium levels were significantly higher in those which did not survive after the glucose solution than the plasma potassium levels of the controls or the adrenalectomized group protected with epinephrine. These results suggest that the rapid rise in plasma potassium concentration probably was responsible for the mortality of the unprotected adrenalectomized groups of rats.

Demedullated groups. Greep and Deane(5) presented histochemical evidence (presence of lipid droplets, and ascorbic acid granules) of the functional status of the regenerated adrenal cortex of demedullated rats. This was confirmed by us in the present study. A significant fall in the number of circulating eosinophiles 180 minutes after an injection of

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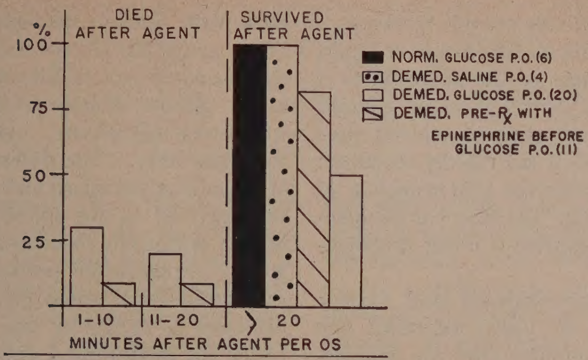

MEAN PLASMA LEVELS OF GROUPS ABOVE

K meq/l	5.4	4.9	5.2	4.6	4.2	3.3	3.4	3.5
±	.26		.97		.19	.15	.13	.09
Glucose mg %	130	240	157	297	230	73	321	143

FIG. 2. Protection of demedullated rats with epinephrine pretreatment against mortality and hyperkalemia following 2 ml of a 50% glucose solution *per os*. No. of rats in each group is in the parentheses. ± is the std error of the mean.

epinephrine in demedullated rats has been reported by Dury(8). This is further evidence of the functional secretory status of the regenerated adrenal cortex. The plasma potassium concentration, however, in demedullated rats is significantly lower than in normal or adrenalectomized rats(3). These points must be considered in the interpretation of the results obtained with the demedullated groups.

The mortality of demedullated rats after the administration *per os* of 2 ml of a 50% glucose solution and the protection which epinephrine pretreatment gave to these rats is shown in Fig. 2. Fifty percent of a group of demedullated rats survived the administration of the glucose *per os*. Pretreatment with a single injection of epinephrine 60 minutes before the administration of the glucose solution protected the demedullated rats as it had protected the adrenalectomized rats. The difference in the percent survival between adrenalectomized and the demedullated groups after the glucose solution was given may be explained on the basis of the points mentioned above. Although the plasma potassium concentration of the demedullated rats increased after the administration of the glucose solution, it did not reach a mortal level since these rats had initially a low potassium concentration. Secondly, since the regenerated adrenal cortices have been shown to be functional, demedullated rats would not be unduly sensitive to a rise in plasma potassium concentration a little above the "normal" level.

Discussion. The experiments presented here showed that adrenalectomized rats were extremely sensitive to a dose of glucose administered directly into their stomachs. The factor apparently responsible was the rapid rise in the plasma potassium concentration which followed this procedure. In previous experiments it had been shown that the adrenal cortex and extracts of the adrenal gland protected animals from potassium toxicity. In these experiments it was shown that epinephrine protected the adrenalectomized rats, whereas pretreatment with lipo-adrenal extract had not protected these animals. Additional evidence that a functional adrenal cortex in the absence of the adrenal medulla was inadequate protection against the rapid mobilization of plasma potassium is indicated by the results obtained from the experiments with the demedullated groups of rats.

Recently, Dury(3) showed that epinephrine must be considered a homeostatic agent in the regulation of the plasma potassium level. The

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experiments reported here provide further evidence of the physiological function of epinephrine in the adjustment of the organism to a situation of "stress" which involved a rapid change in the concentration of a blood electrolyte. The source of the rapidly mobilized plasma potassium after the administration of glucose *per os* and the "blocking" of this phenomenon with epinephrine is under investigation.

Summary. A hyperkalemia and cardiac standstill resulting in 100% mortality was induced within 20 minutes after the administration of 2 ml of a 50% glucose solution into the stomachs of adrenalectomized rats. Pretreatment with epinephrine 60 minutes before the administration of the glucose solution protected the adrenalectomized rats. Pretreatment with lipo-adrenal extract 120 minutes before giving the glucose solution did not protect the adrenalectomized rats. The admission of the glucose solution to demedullated rats with functional adrenal cortices resulted in a 50% mortality. The difference in mortality of the adrenalectomized and demedullated rats was ascribed to the initial relatively "low" plasma potassium concentration of the latter group. Epinephrine pretreatment protected the demedullated group too. These data are discussed as indicating that epinephrine protected the experimental groups of rats by inducing a lower than "normal" plasma potassium concentration and "blocking" a change to supranormal levels after the glucose *per os*.

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Effect of Iodide, Thiouracil and Thyroxine on the Disappearance of Thyroidal I^{131}. (18723)

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A direct method for determining *in vivo* the thyroidal secretion rate in rats has been described(1). The method consists of injecting a tracer dose of I^{131} and making daily observations over the thyroid area in terms of counts per second for a period of about 2 half-lives of I^{131} (16 days). These observations, when plotted on semilog paper, can be fitted to a straight line, and from this line the proportional rate of diminution of thyroidal I^{131} can be determined and expressed as per cent per day. This proportional secretion rate, when calculated in terms of μg of thyroxine secreted per day, agrees with calculations made by other methods. Furthermore, it has been shown that hypophysectomy, which is known to depress thyroidal activity, reduced the secretion rate to one-twelfth that of normal animals(2). Further proof that this method measures hormonal secretion was obtained by determining the effect of thiouracil, thyroxine and sodium iodide on the disappearance of thyroidal I^{131}.

Material and methods. Weanling male rats (Sprague-Dawley) were used under conditions as described previously(2). After 2 weeks on an iodine deficient diet, the animals were injected subcutaneously with a tracer dose of 1 μc of radio-iodine, and observations were made daily as described. After the 48-hour count was obtained, the animals were separated into four groups: (1) controls; (2) 1% thiouracil in the drinking water; (3) 1% sodium iodide in the drinking water; (4) 10 mg d-l-thyroxine injected daily by subcutaneous route. Observations were continued for 16 days, and the proportional rates of disappearance of thyroidal I^{131} were determined. Three experiments, performed at different times of the years, are shown in Table I.

Results. As shown in Table I, the normal rate of hormonal secretion was between 8 and 9% per day with close agreement in the 3

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1. Albert, A., *Endocrinology*, 1951, v48, 334.

2. Randall, R. V., Lorenz, N., and Albert, A., *Endocrinology*, 1951, v48, 339.

TABLE I. Proportional Rate of Disappearance of Thyroidal I^{131} (Mean ± Sem.).

		Controls		Sodium iodide		Thiouracil		Thyroxine	
Exp.		No.	%/day	No.	%/day	No.	%/day	No.	%/day
1	9/1950	9	9.33 ± .49	8	4.39 ± .58	7	48.89 ± 5.80	7	4.35 ± .42
2	11/1950	9	9.25 ± .39	9	4.40 ± .45	7	56.25 ± 4.69	7	3.84 ± .39
3	1/1951	6	8.03 ± .97	7	11.52 ± 1.86	6	44.14 ± 5.00	7	4.71 ± .54

separate experiments. For thiouracil the average rate was 50% per day and for thyroxine, 4.3% per day, again with little variation among the three separate experiments. However, in the iodide experiment, the rates obtained in the first 2 experiments were distinctly different from the control rate but there was no significant change from the control rate in the third experiment.

Discussion. The results obtained add further support to the proposition that the *in vivo* method as described measures hormonal secretion and does so quantitatively. First, it is known by other tests that administration of thyroxine suppresses endogenous thyroxine secretion, and in the present experiments the suppression was more than half the control rate ($P \leqq 0.02$). Secondly, the proportional rate of disappearance (50% per day) of thyroidal I^{131} after administration of thiouracil coincides exactly with Astwood's(3) value of 50% per day for the disappearance of total thyroidal I^{127} after the administration of thiouracil.

The effect of iodide is, however, somewhat puzzling, since iodide supposedly does not affect normal thyroidal secretion. In Experiments 1 and 2, however, significant depression occurred ($P \leqq 0.01$), whereas in the third experiment, no significant change from normal was present ($P = 0.2$). These divergent results may be due (1) to the fact that iodine deficient rats are not normal rats, (2) to a seasonal effect or (3) to unknown causes. It is also puzzling that the administration of thyroxine, although inducing a significant depression of endogenous hormonal secretion, did not suppress thyroidal activity as severely as hypophysectomy did. The dose of thyroxine used was 2 to 4 times the normal daily output of thyroxine and should have induced a complete inhibition of thyroidal secretion, if its action is solely via thyrotropin inhibition. Finally, thiouracil increased the disappearance rate of thyroidal I^{131} sixfold, indicating that an immediate acceleration of TSH secretion occurred, or that a potentiating process involving TSH was invoked, or that some factor inhibiting thyroidal secretion was removed. If the effect of thiouracil were simply one of blocking further hormonal synthesis while allowing preformed hormone to be secreted at a normal rate, no effect on the disappearance rate of thyroidal I^{131} should have been noted.

Summary. The effect of thiouracil, iodide and thyroxine on thyroidal secretion was determined by an *in vivo* method utilizing I^{131}. Thiouracil accelerated thyroidal secretion sixfold; thyroxine inhibited it, and sodium iodide induced either no change or a depression of secretory rate.

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Effect of Hypophysectomy on the Intrathyroidal Metabolism of I^{131}. (18724)

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Morton *et al.*(1) reported that hypophysectomy induced a qualitative defect in the treatment of iodine by the thyroid in that the conversion of iodine to thyroxine was definitely limited or almost inhibited. However, it would appear more likely from other work on the pituitary-thyroid system that the defect induced by hypophysectomy would be quantitative rather than qualitative. If the latter were true, then a longer time interval would be needed to manifest the existence of a slow rate of conversion of diiodotyrosine to thyroxine in hypophysectomized rats than in normal rats. Also improved methods of analysis would be needed to detect any small differences in the proportions of thyroidal diiodotyrosine and thyroxine at varying intervals in normal and hypophysectomized rats. Consequently, the experiments of Morton *et al.* were repeated with two modifications: (1) observations were extended over a longer time interval, and (2) methods of analysis which we believe represent advances over those used by Morton *et al.* were employed.

Material and methods. Weanling Sprague-Dawley male rats were used after they had been maintained for 2 weeks on an iodine deficient diet under conditions described previously(2). On the sixth day after hypophysectomy, the hypophysectomized and the control rats were injected with a tracer dose of 10 μc of radioiodine. Groups of animals were killed at 3, 6, 24, 48, 72, 96 and 144 hours after the injection of the tracer dose. The thyroids were removed, and the radiation was determined in a gamma ray counter and expressed as per cent of dose by counting simultaneously an aliquot of the administered dose. Each thyroid was then homogenized in a few ml of 10% trichloracetic acid in an all-glass homogenizer. The homogenate was then centrifuged, and the supernatant liquid was transferred to a 15 ml centrifuge tube. The precipitate was then washed twice with 5 ml of trichloracetic acid. After centrifugation, the supernatant solutions were combined and the radiation in the acid-soluble and acid-insoluble fractions was determined. The percentage of radioactivity in each fraction was then computed by dividing the radioactivity in each fraction by the sum of the activity of the two fractions. The acid-insoluble fraction consists of protein-bound I^{131} and any free thyroxine that may be present. The acid-soluble fraction contains, among other things, inorganic iodide and any free diiodotyrosine that may be present. The protein-bound or acid-insoluble fraction was emulsified in 10 ml of water in a 15 ml centrifuge tube, and 1% sodium hydroxide was added until the pH of the emulsion was just alkaline to phenolphthalein. One ml of 2 N sodium hydroxide solution was then added, and the tube was placed in an incubator at 95°C for 8 hours. After cooling, the solution was neutralized with trichloracetic acid. To each hydrolyzed sample was added sufficient saturated sodium chloride solution to make a 1% solution, 0.1 ml of human albumin solution (Cutter Laboratories) and 1 mg each of sodium iodide, diiodotyrosine and thyroxine as carriers. The procedure for obtaining and computing the acid-soluble and acid-insoluble fractions as described previously was repeated.

The efficiency of this method, both before and after hydrolysis of thyroid tissue, was determined by performing recovery experiments with radioactive iodide, radioactive diiodotyrosine and radioactive thyroxine. Ninety-three per cent of the radiothyroxine added to thyroid homogenate was recovered in the acid-insoluble fraction, and 96 and 91% respectively of iodide and diiodotyrosine were recovered in the acid-soluble fraction. Ninety-two per cent of radiothyroxine added after hydrolysis was recovered in the acid-

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2. Randall, R. V., Lorenz, Nona, and Albert, A., *Endocrinology*, 1951, v48, 339.

TABLE I. Effect of Hypophysectomy on Intrathyroidal Metabolism of I^{131}.

	Hr after injection						
	3	6	24	48	72	96	144
Total I^{131} % uptake							
Normal	53.65 (7)*	60.96 (2)	55.07 (2)	77.27 (6)	59.39 (2)	52.98 (2)	44.08 (7)
H†	4.29 (8)	9.06 (3)	25.99 (3)	29.76 (9)	28.82 (4)	33.59 (4)	18.66 (5)
% acid-insoluble							
Normal	96.95 (4)	94.43 (4)	95.67 (2)	95.25 (2)	97.58 (2)	95.75 (2)	
H	82.55 (8)	90.82 (8)	93.77 (3)	94.79 (4)	97.51 (4)	95.29 (4)	
% acid-soluble							
Normal	3.06 (4)	5.58 (4)	4.34 (2)	4.75 (2)	2.42 (2)	4.25 (2)	
H	17.46 (8)	9.18 (8)	6.23 (3)	5.21 (4)	2.50 (4)	4.71 (4)	
After hydrolysis							
% acid-insoluble							
Normal	16.80 (9)	20.93 (4)	38.34 (1)	43.49 (6)	42.51 (2)	45.99 (2)	52.71 (7)
H	15.74 (13)	10.98 (8)	9.87 (3)	22.44 (8)	15.14 (4)	17.49 (4)	25.04 (4)
% acid-soluble							
Normal	83.20 (9)	79.08 (4)	61.66 (1)	56.51 (6)	57.49 (2)	54.02 (2)	47.29 (7)
H	84.26 (13)	89.01 (8)	90.13 (3)	77.57 (8)	84.86 (4)	82.51 (4)	74.96 (4)

* Numbers in parentheses are numbers of animals.
† H = hypophysectomized.

insoluble fraction, and 97 and 90% of iodide and diiodotyrosine respectively added after hydrolysis were recovered in the acid-soluble fraction.

Results. The results are shown in Table I, which gives average values for each group of rats and, in parentheses, the number of animals on which each mean value is based. It is evident from this table that, while substantially all of the I^{131} became protein-bound within a day in the hypophysectomized animals, the rate and extent of the protein-binding was somewhat slower than in normal animals. The most significant observation was in the behavior of the acid-soluble and acid-insoluble components after hydrolysis of the protein-bound I^{131}. In the normal animals, there was a progressive increase in the acid-insoluble fraction until more than half of the radioiodine was in the form of thyroxine. In the hypophysectomized animals, there was a much smaller increment in the thyroxine fraction after 144 hours. Concomitantly, the acid-soluble fraction in normal animals decreased rapidly over the period of 144 hours, whereas in hypophysectomized animals, there was a smaller decrease.

Discussion. The present results indicate that hypophysectomy induces a quantitative rather than a qualitative defect in the intrathyroidal metabolism of I^{131}. In hypophysectomized rats the proportion of acid-soluble I^{131}, assumed to be the precursor of thyroxine, decreases slightly over a period of 144 hours but the slope* of this decrease (0.092% per hour ± 0.026) is significantly different from zero. This would indicate that hypophysectomized animals are capable of converting iodide into thyroxine but at a rate which is only a fraction that of normal animals.

Summary. Hypophysectomy reduced but did not abolish the over-all conversion of I^{131} into labeled thyroxine.

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* The curves were analyzed by Dr. Joseph Berkson, to whom we are indebted.

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Vascular Participation in Tumor Slice Metabolism.* (18725)

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The metabolism of tumor tissues has been the subject of numerous investigations, particularly in regard to oxygen consumption(1). In an effort to more closely correlate metabolic activity with the actual cancer cell content of a tumor mass Chalkley introduced a point pattern method of measurement(2). While good correlation is found between the per cent of epithelial cells and certain enzyme activities by such technics, this does not exclude the possibility that stromal elements are also participating in the respiration in some constant relationship with the tumor cells. More recently we have introduced the use of tetrazolium chloride for the measurement of the metabolism of tissue slices(3,4). This procedure has the advantage that the red insoluble formazan, formed as the result of the dehydrogenase activity of the tissues, is deposited at the site of reaction. Frozen section examination of the tissues after incubation of the tissue slice in tetrazolium chloride will then allow visualization of the cells involved (5-7). Advantage was taken of this phenomenon to study the participation of stromal and cancer cells of tumors as contrasted with such activity of normal tissues. Such activity could also be characterized by the use of selected enzyme inhibitors.

Our findings would indicate that in spontaneous breast carcinoma, arising in C3H and CFW mice and in a transplantable mammary carcinoma grown in C57 black mice, no formazan was deposited in the tumor cells, themselves; but rather was limited to the endothelial cells of the fine capillary network within the tumor and also to some extent in the adjacent connective tissue stroma. Similar findings were also noted in methyl cholanthrene induced epidermoid carcinoma of the skin of mice. This reaction was not inhibited by fluoride, malonate or azide.

Materials and methods. The tumors included in this study were spontaneous mammary carcinomas arising in female mice of the C3H and CFW strains, as well as transplantable mammary carcinoma implanted subcutaneously in CFW or C57 black mice. The control tissues included kidney, liver, adrenal, lung, normal mammary gland and skin. The animals were sacrificed by rapid crushing of the cervical cord and the tissues to be studied were immediately dissected out and sectioned into slices approximately 1 mm in thickness. In the case of the tumors, care was taken to employ only those areas which showed no gross evidence of necrosis. Five determinations were usually run on each tissue, and the determinations were run in duplicate except in the case of the adrenal where the small size of the organ made this impossible. Representative slices were placed in the incubation mixtures, each of which contained 3 ml of a 1% aqueous solution of 2,3,5-triphenyltetrazolium chloride (*TTC*) buffered with phosphate buffers to pH 7.2. The different mixtures had in addition one ml of (a) 0.9% sodium chloride, (b) 10^{-3} M sodium fluoride, (c) 10^{-3} M neutralized malonic acid, (d) 10^{-3} M sodium azide and (e) 2×10^{-4} M neutralized succinic acid. The tubes were placed in a rack in an incubator, maintained at 37°C

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* Aided by grants from Damon Runyon Memorial Fund for Cancer Research, to the New York Medical College and Leukemia Research Foundation.

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and subjected to continuous gentle agitation for a period of one hour. At the end of this time they were removed, the solutions decanted and the tissues fixed in 10% formalin. If kept in the dark, the fixed tissues will be suitable for quantitative analysis or microscopic examination for at least a week. After fixation, the tissues were sectioned for histological examination on the freezing microtome and then the remaining tissue and the unsectioned duplicates were subjected to acetone extraction of the reduced TTC, the concentration of which is determined in μg per mg of acetone dried tissue according to the technic previously described(3,4). The sections for histological examination were studied both with and without counterstaining with polychrome methylene blue. If the sections are mounted in glycerine and kept in the dark they may be considered as semipermanent. However, it was our practice to examine such sections as soon as they were cut. In many instances the histological examination was made within a few hours of the time the animal was sacrificed.

Results. This report is primarily concerned with the observations on the tumor tissues. The findings on the other tissues will be discussed only in so far as they provide a control for pertinent observations made on the tumors.

The spontaneous mammary tumors arising in the C3H and the CFW strains and the transplantable mammary carcinoma behaved in a similar fashion in regard to the quantitative determination and the histochemical appearance. For this reason no differentiation between them will be made in the following discussion of the results.

After incubation the tumor slices in each of the different solutions were seen to be diffusely red in color. Frozen section examination revealed that the tumor cells, themselves, were free of any intracellular formazan deposition. In contrast, the vascular structure of the tumor was sharply outlined by the deposition of elongated crystals of reduced TTC within the cytoplasm of the endothelial cells comprising the capillaries of the tumor (Fig. 1). In counterstained sections the intimate relationship of the capillaries to the individual tumor cells is clearly demonstrated and corresponds to the descriptions of Algire and Chalkley of tumor vasculature(8). Most of the intrinsic vessels consist of naked endothelial tubes closely applied to the tumor cells in a fashion which is suggestive of the type of arrangement obtaining in endocrine glands. However, there is no indication that the vascular channels are discontinuous or sinusoidal in character. Perivascular fibroblasts noted around larger vessels also show intracellular deposits of reduced TTC. In addition a moderate amount of extracellular amorphous formazan deposition was scattered throughout the section. This latter finding is most marked in the tissues incubated in the presence of succinate. The qualitative histochemical picture was found to be essentially the same in the tissues which had been incubated in each of the different solutions, *viz.*, the formazan deposition was confined almost exclusively to the endothelial cells of the tumor capillaries, perivascular fibroblasts and stromal macrophages. No evidence of inhibition of such staining was observed to occur in the presence of the fluoride, malonate or azide, a finding confirmed by quantitative determinations of the μg of TTC reduced per mg of acetone dried tissue.

In contrast to the intracellular reduction of TTC by tumor capillaries and stroma such activity was minimal or absent in the vessels and stroma of normal mammary parenchyma, adrenal cortex and medulla, kidney, liver, skin and lymph node.

It was noted, however, that in the region of the precapillary sphincters there were perivascular cells which reduced the TTC. Some formazan deposition may also occur in inflammatory foci of the slices incubated in the TTC with saline or succinate. However, when these tissue slices were incubated in the presence of fluoride, malonate or azide no TTC reduction by the vessels or stroma was observed.

Discussion. The observation that the endothelial and stromal cells of these tumors actively reduce TTC is indicative of a unique stromal parenchymal relationship which has

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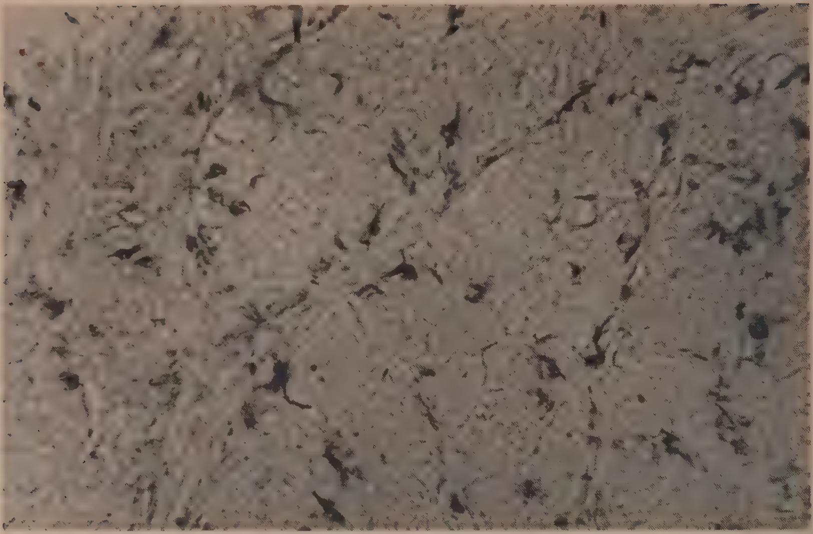

FIG. 1. Spontaneous breast carcinoma; C3H mouse, after incubation in TTC for 1 hr. 440×.

not been observed thus far in a wide variety of normal tissues. It should also be mentioned at this time that a similar although not as clearly defined phenomenon occurs in human cancer tissues; indication of the general participation of this type of activity in malignant tumors. Details of these studies will be reported shortly.

As connective tissue and stromal vessels usually show minimal oxygen consumption and dehydrogenase activity it has been tacitly assumed in the past that the larger part of such activity by a tissue slice represented the metabolic activity of the parenchymatous elements. While this is apparently true for normal tissues, the present observation would place serious limitations on such an assumption in regard to cancer tissues.

In consideration of an altered enzyme activity in tumor vessels, it should be noted that a marked diminution in alkaline phosphatase has been shown to occur in these vessels(9). Manheimer and Seligman point out that while ordinary histological methods cannot differentiate young vessels in malignant tumors from those in neighboring tissues, alkaline phosphatase determinations suggest a physiological difference. They call attention to the destructive action of bacterial polysaccharides on animal and human tumor vasculature as contrasted to the minimal effect on the vessels of normal tissues.

On the basis of these observations and our own, it would appear that the tumor cells and their associated stroma constitute a unique biochemical and biological entity. Further evaluation of this assumption appears worthy of more intensive investigation.

Summary. Tissue slices from mammary carcinoma and diverse normal tissues of mice of different strains were incubated in TTC solutions with and without fluoride, malonate, azide and succinate. Frozen section examination after such treatment revealed formazan deposition in the vasculature and stroma of the tumor but none in the tumor cells, themselves. This manifestation of dehydrogenase activity in the stromal vessels was not inhibited by fluoride, malonate or azide; a finding confirmed by quantitative determinations of μg of TTC reduced per mg of acetone dried tumor tissue. No such stromal dehydrogenase activity was found in slices of normal mammary parenchyma, adrenal cortex or medulla, kidney, liver, skin or lymph node. These findings are discussed in terms of a unique function of tumor capillaries.

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Effect of Feeding Penicillin on the Life Cycle of the Chick.* (18726)

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Stokstad and Jukes(1) and Whitehill *et al.*(2) reported that aureomycin, streptomycin, penicillin, and sulfasuxidine promoted the growth of chicks adequately supplied with vit. B_{12}. Reed and Couch(3) failed to obtain an increase in the growth of chicks kept on sand litter by adding an APF concentrate, which contained only vit. B_{12}, to an all-vegetable-protein diet, but obtained a very significant increase in chick growth by adding an APF obtained from *Streptomyces aureofaciens* fermentation. The type of dietary carbohydrate has been reported to influence the microflora in fecal droppings from laying pullets(4). These workers found that dextrin and lactose stimulated the development of coliform bacteria, whereas, sucrose had a depressing effect on the fecal coliforms. Penicillin and terramycin have been reported to have an inhibitory effect on *Clostridium perfringens* in the intestinal tract of turkeys(5). These workers stated that it appeared possible that these antibiotics might promote growth through such inhibition.

With these reports in mind it appeared desirable to determine the effect of feeding penicillin on growth, egg production, hatchability, and on the fecal microflora of the birds fed this antibiotic.

Experimental. Crossbred chicks (N.H. × S.C.W.L.) used in these studies were obtained from hens which had been fed a practical all-mash diet(6). This diet has been used to produce B_{12}-depleted chicks and has been shown to produce eggs with yolks of low B_{12} content(7). The chicks were kept in batteries with raised screen floors with feed and water supplied *ad libitum*. The birds were weighed at weekly intervals during an experimental period of 32 weeks. Cockerels were removed at the end of the 10th week. Pullets were transferred to individual laying cages at 9 weeks of age. The management and experimental procedures with regard to the care and handling of the pullets, insemination, incubation, and embryonic mortality records were the same as those previously reported by Couch *et al.*(8), and Olcese, Couch, and Lyman(9). The basal diet (G-1) used in this study was the same as that reported earlier by Couch and Olcese(10). One hundred chicks were randomized into 4 groups of 25 chicks each. The first and second groups were fed the basal diet. Each chick in the second group received an injection of 1 μg of crystalline vit. B_{12} per chick per week into the pectoral muscle. Groups 3 and 4 were fed the basal diet supplemented with 33 mg of procaine penicillin (G) per kg of diet. Each chick in Group 4 was injected with 1 μg of crystalline vit. B_{12} weekly.

Six cockerels from each group were killed

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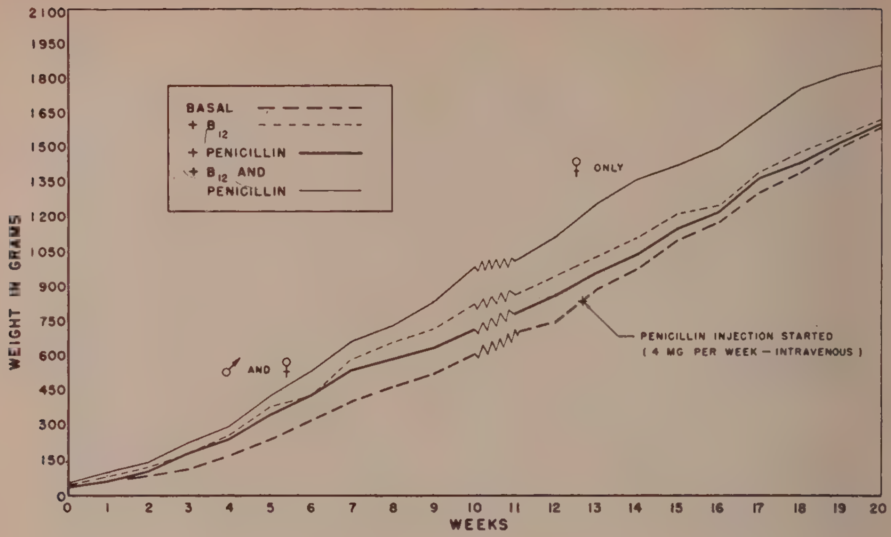

FIG. 1. Effect of feeding penicillin and injecting B_{12} on weights of crossbred chicks.

at the end of the 10th week and the livers and kidneys were removed for vit. B_{12} determination. Samples of liver and kidney tissue were prepared for assay according to the method of Couch and Olcese(10). Eggs were collected for B_{12} determination during the 26th and 27th weeks of the test. Egg yolks from each group were pooled and an aliquot of the yolks was prepared for vit. B_{12} assay according to the method of Halick and Couch(7). The vit. B_{12} content of the tissues and egg yolks was determined according to the method of Skeggs *et al.*(11). Fecal droppings for bacteriological analysis were collected on clean waxed paper between 5:00 and 6:00 a.m. at weekly intervals from the fifth through the 15th and from the 18th through the 30th weeks. Droppings from each group were thoroughly mixed with 50 ml of sterile 0.1% tryptone solution in a 100 ml dilution bottle until all visible clumps were dispensed. Serial decimal dilutions were made in sterile 0.1% tryptone solution up to 10^{-10} dilution. Inoculations from such dilutions were made into the sterile media described below. The thioglycollate medium (Baltimore Biological Laboratories) was used for making total dilution counts. The enterococci dilution counts were made by using the "SF" broth of Hajna and Perry(12). Difco eosin methylene blue agar ("EMB") was used for making coliform plate counts. Yeast counts were made by using the potato-dextrose agar acidified to pH of 3.5 with citric acid (13). Carrot-liver extract ("CL") agar shake tubes were used for counting lactic acid bacteria(14). Plate counts of gram-negative bacteria were made with crystal violet agar. Penicillin resistant bacteria were determined by plating in nutrient agar containing 100 μg of procaine penicillin (G) per ml. The thioglycollate medium, Difco eosin methylene blue agar, crystal violet agar, and penicillin medium were incubated at 37°C for 48 hours. "SF" broth was incubated at 45°C for 3 days. Potato-dextrose agar was incubated at room temperature for 5 days. Carrot-liver extract agar was incubated at 37°C for 72 hours. All media were plated and tubed in duplicate with

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TABLE I. Effect of Feeding Penicillin and Injecting Vit. B_{12} on Egg Production, Hatchability and B_{12} Content of the Liver and Kidney Tissues, and Egg Yolk.

Supplement to basal diet	Egg production, %	Hatchability, %	µg B_{12} per 100 g Liver	µg B_{12} per 100 g Kidney	mµg B_{12}/g egg yolk
None*	29	0	11.7	11.9	1.7
1 µg B_{12} inj./bird/week	38	72	15.2	16.5	3.4
Penicillin (33 mg/kg)	22	39	13.2	11.6	2.1
Penicillin (33 mg/kg) + 1 µg B_{12} inj./bird/week	50	80	17.7	18.4	3.1

* Four mg penicillin was inj. intrav. from 14th through 32nd week.

the exception of thioglycollate and "SF" broth. The latter were run in a series of 5 tubes per dilution. For convenience in handling, each individual count was converted to its respective logarithm to the base 10 (the mantissa was carried out to the second place). An analysis of variance for counts obtained on each medium was made, and the F value (ratio of appropriate mean square values from an analysis of variance table) was determined. Least significant differences (LSD) were calculated where the F value was significant at the 1 or 5% level of probability.

Results and discussion. A summary of results up to the time the males were removed (10 weeks) shows that the addition of penicillin to the basal diet resulted in increased growth (Fig. 1). The injection of vit. B_{12} resulted in a greater increase in growth than did addition of penicillin, though this difference did not appear until the seventh week. When the birds received both vit. B_{12} and penicillin the increase in weight was greater than either given alone.

Due to the excessive mortality in the basal group it seemed advisable to modify the experiment at the end of the 14th week. Since stimulation has been shown by incorporating penicillin in the diet it seemed worthwhile to evaluate the influence of intravenous injection of this antibiotic. The basal birds were, therefore, given an intravenous injection of 4 mg of procaine penicillin (G) at weekly intervals for the duration of the experiment. This break in continuity necessitated treating the experimental data as two separate experiments. Growth of these birds was improved by such injection and the weight obtained at the end of the 20th week was almost identical with that of birds receiving penicillin and of those receiving B_{12} throughout the period. All birds started to lay at 19-23 weeks of age. Egg production and hatchability data were collected for a period of 12 weeks (Table I). Birds fed penicillin and injected with vit. B_{12} had the highest egg production during this period. Couch *et al.*(6) have shown that vit. B_{12} is necessary for egg production.

No live chicks were obtained from eggs laid by pullets from the basal group which received an intravenous injection of penicillin after the 14th week. Typical symptoms of vit. B_{12} deficiency, as described by Olcese *et al.*(15) were observed in these embryos. In contrast, 39% hatchability was observed in eggs from birds fed penicillin continuously. It is possible that the feeding of the antibiotic continuously led to the establishment of a more favorable microflora which may have synthesized vit. B_{12} at a sub-optimal level. The per cent hatchability of eggs from pullets injected with B_{12} and those injected with B_{12} and fed penicillin is considered to be within the normal range.

The vit. B_{12} content of the liver and kidney tissues from cockerels at 10 weeks of age was increased by injecting the vitamin into the birds, but apparently was not increased to any appreciable extent by feeding the antibiotic (Table I). This is in agreement with the work of Couch and Olcese(10).

The injection of vit. B_{12} into the hens increased the concentration of this vitamin in the egg yolk (Table I). There appears to be little difference in the B_{12} content of the eggs from the birds receiving the two types of penicillin treatment. It is possible that vit. B_{12} was not the limiting factor influencing the

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15. Olcese, O., Couch, J. R., Quisenberry, J. H., and Pearson, P. B., *J. Nutrition*, 1950, v41, 423.

TABLE II. Summary of Statistical Analysis of Data from Fecal Droppings of Chickens up to 14 Weeks of Age.

Media and type of count	Avg				F-value
	Basal	B_{12}	Penicillin	Penicillin + B_{12}	
Thioglycollate (Total dilution count)	8.11*	7.71	8.76	8.91	5.72†
Carrot-liver (Lactics)	7.81	7.15	7.79	7.99	1.70
"SF" broth (Enterococci)	4.43	4.55	5.87	6.10	11.96†
Penicillin (Resistant penicillin)	2.96	4.07	6.16	5.96	8.44†
Eosin methylene blue (Coliform)	7.01	6.68	7.89	7.92	2.84
Crystal violet (Gram-negative)	7.36	6.39	7.50	7.40	1.92
Potato-dextrose (Yeast)	3.35	3.42	4.04	4.44	1.71

* Avg log of number of bacteria per g feces (wet wt).

† Significant at 1% level (probability of chance occurrence less than 1%).

TABLE III. Summary of Statistical Analysis of Data from Fecal Droppings of Chickens from 18 to 30 Weeks of Age.

Media and type of count	Avg				F-value
	Basal	B_{12}	Penicillin	Penicillin + B_{12}	
Thioglycollate (Total dilution count)	9.19*	9.03	9.67	9.72	6.65†
Carrot-liver (Lactics)	8.66	8.60	8.72	8.51	.40
"SF" broth (Enterococci)	5.25	4.88	5.11	5.54	.97
Penicillin (Resistant penicillin)	5.13	4.44	5.35	6.05	8.40†
Eosin methylene blue (Coliform)	6.81	6.68	6.98	7.18	1.19
Crystal violet (Gram-negative)	6.57	6.39	6.80	6.95	2.22
Potato-dextrose (Yeast)	3.15	2.15	4.27	4.92	69.27†

* Avg log of number of bacteria per g feces (wet wt).

† Significant at 1% level (probability of chance occurrence less than 1%).

hatchability in the latter groups. This observation is in agreement with Halick and Couch(7) who reported that eggs containing as little as 1.7 mμg of B_{12} per g gave normal hachability.

The feeding of penicillin produced an increase in the total dilution count (thioglycollate), enterococci ("SF" broth), and an increase in the penicillin resistant organisms (Table II), from the 5th through the 14th week of the test.

Apparently there was some adjustment in the intestinal microflora after the 14th week of test (Table III). The difference in the enterococci counts, observed as being traceable to feeding penicillin was not significant from the 18th through the 30th weeks of test. The injection of the antibiotic had a rather definite effect on the number of penicillin resistant organisms and on the number of yeasts in the intestinal tract.

It seems likewise evident that the injection of this antibiotic stimulated the growth of these birds through a shift in the microflora of the gastrointestinal tract.

Summary. 1. The feeding of penicillin stimulated the growth of birds fed an all-vegetable-protein diet. 2. Injection of the antibiotic into the birds fed the basal diet stopped the mortality and appeared to stimulate the growth. 3. Eggs were obtained from all groups. Pullets fed penicillin and injected with vit. B_{12} had the highest egg production. Hatchability of eggs from birds injected with B_{12} was within the normal range. No live chicks were obtained from pullets injected with penicillin from the 14th week. A 39% hatch was obtained from the eggs of pullets fed penicillin continuously. The B_{12} content of the liver and kidney tissues and of the egg yolks was directly related to the injection of the vitamin into the birds. 4. Feeding of penicillin caused a significant increase in the total number of intestinal micro-organisms as indicated by thioglycollate counts. Penicillin likewise caused a significant increase in the number of enterococci of the intestine during the first 14 weeks of the experiment. The administration of penicillin either orally or intravenously resulted in a marked increase in the number of penicillin resistant micro-organisms in the intestinal tract.

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Inhibition of Growth of C3H Mice by Ethionine and its Alleviation by Methionine or Choline.* (18727)

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In a recent communication we reported(1) that either optical isomer of ethionine (S-ethyl-homocysteine) inhibited the growth of albino rats which were maintained on a complete casein diet. The inhibition could be alleviated by either isomer of methionine or by choline, but not by cystine, homocystine, or cystathionine. The observation that choline was effective suggested that ethionine was interfering with the economy of the labile methyl group. Further studies employing ethionine labeled with C-14 in the ethyl group revealed that the ethyl group carbon was incorporated into the molecule of choline and creatine of rat tissues. This suggested the possible "transethylation" of ethionine(2). Similar conclusions were made in experiments employing S^{35}-ethionine(2). It appeared of interest to extend these observations to other species, such as the mouse.

Experimental. Weanling mice of C3H strain, inbred by brother-sister method, born and raised in the laboratory, were used. At the age of 23 days the males and females were separated and placed in separate cages, 3 mice per cage. A total of 66 mice were used. The composition of the basal diet was as follows: Labco casein 30; corn starch 36; sucrose 15; Crisco 10; inorganic salts (USP XII No. 2) 4; cod liver oil 5%. Per kg this diet was fortified with the following vitamins: thiamin hydrochloride 20; riboflavin 20; calcium pantothenate 50; pyridoxine hydrochloride 20; nicotinamide 20; folic acid 5; inositol 1000; p-amino-benzoic acid 1000; biotin 1.0; alpha-tocopherol 300 mg; and vit. B_{12} 150 μg. No choline was added to the basal diet. The preparation and analytical purity of ethionine, methionine, cystine, and homocystine used in the present study were previously described (1). The food and water were fed *ad libitum*, the mice were weighed twice a week, and the food consumption was recorded. At the end of the experimental period the livers of mice in each group were pooled, and their lipid content was determined gravimetrically(1). The supplements fed with the basal diet are indicated in Table I.

Results. Since the incorporation of 0.5% of DL-ethionine into the diet proved fatal to the growing mice within 24 to 48 hr after its ingestion, the concentration was reduced to 0.25%. As will be observed from the data in Table I, this amount retarded or inhibited the growth. Supplementation of this ethionine-containing diet with 0.25% of methionine per-

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* Aided by a grant from National Cancer Institute, National Institutes of Health, U.S.P.H.S., Bethesda.

1. Stekol, J. A., and Weiss, K., *J. Biol. Chem.*, 1949, v179, 1049.

2. Stekol, J. A., and Weiss, K., *J. Biol. Chem.*, 1950, v185, 577.

TABLE I. The Effect of Ethionine in Mice Fed a Complete Casein Diet.

Mice group‡	Initial wt, g	Final wt, g	Food per day, g	Gain per day, g	Days on diet	Wet liver lipids, %	Supplements, %
1 ♂	10.8	19.4	1.8	.41	21	4.7	0
2 ♀	10.8	16.8	2.3	.30	21	4.7	0
3* ♂	11.2	10.5	1.0	–.03	21	4.0	.25 DL-ethionine
4† ♀	10.6	12.5	1.0	.09	21	4.0	.25 ”
5 ♂	11.7	17.5	1.6	.32	21	5.84	.25 ” , .25 DL-methionine
6 ♀	12.3	17.2	1.6	.23	21	5.84	.25 ” ”
7 ♂	9.3	8.0	2.0	–.10	14		.25 ” , .5 choline chloride
	8.0	15.0	2.0	.37	19		.25 ” , 1. ”
8 ♂	9.2	10.2	1.7	.05	22	5.71	.25 ” , .25 L-cystine
9 ♂	10.4	18.0	1.9	.35	22	5.67	.25 ” ” .5 choline
10 ♂	9.6	8.7	1.8	–.06	14		.25 ” , .3 homocystine
	8.7	14.8	2.0	.37	16		.25 ” , 1. choline chloride
11 ♂	10.7	19.0	2.1	.40	21		.25 ” ”

* One mouse died on 19th day.

† Two mice died on 12th day.

‡ Each group consisted of 6 animals from the same litter of inbred mice. All values are average. Data on dead mice were discarded.

mitted resumption of growth, but not quite at normal rate. Addition of 0.5% of choline chloride in lieu of methionine was ineffective (unless fed together with 0.25% of cystine, as in Group 9), while 1.0% of choline chloride completely reversed the inhibition. Neither cystine nor homocystine was effective. No lipid accumulation in the livers of ethionine-fed mice of either sex was noted. Since the ethionine-fed mice showed a reduction in food consumption which, as is well known, might affect the lipid content of the livers, we hesitate to attach unequivocal significance to the lipid data.

Discussion. The growth data obtained on mice are similar to those reported for the rat (1) in that either methionine or choline alleviated the inhibition of growth by ethionine, but that neither cystine nor homocystine was effective. It appears significant that 0.25% of ethionine in a diet which contained 30% of casein (hence, ample methionine) produced such drastic effects on growth of mice, if ethionine is merely an "antimetabolite" of methionine. Of further interest is the fact that the addition of 0.25% of methionine to the 30% casein diet alleviated to a large measure the inhibition by the ethionine. Since choline also completely counteracted the inhibition induced by ethionine, the metabolic disturbance appears to be concerned with groupings common to methionine and choline, namely their labile methyl groups. The incorporation of C-14 or S^{35}-labeled ethionine into various metabolites and tissues of the rat(2) could be duplicated in the mouse(3). These considerations do not exclude the possibility of a direct or indirect incorporation of ethionine *per se* or of only its ethyl group into tissue proteins(1).

Summary. 0.25% of ethionine fed together with a complete 30% casein diet inhibited the growth of weanling C3H mice. The inhibition was alleviated by either choline or methionine, while neither cystine nor homocystine was effective. The observations are discussed in the light of active participation of ethionine or of its ethyl group in metabolic transformations in which methionine and choline methyl groups are normally involved.

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3. Stekol, J. A., and Weiss, K., Unpublished data.

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Comparative Effects of ACTH, Cortisone, Corticosterone, Desoxycorticosterone, Pregnenolone on Growth and Development of Infant Rats.

(18728)

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In a previous paper(1), it was reported that desoxycorticosterone (DCA) injected into newborn rats in daily doses of 0.25 mg did not affect body growth but stimulated eye opening and eruption of teeth. This hastening of development was apparently not mediated through the thyroid gland because the phenomenon occurred despite depression of the gland by means of thiouracil(2). Aqueous adrenal cortical extracts had an effect similar to DCA indicating that the active principle was not solely related to the "salt and water" hormone.

The present report deals with the effects of more recently available hormones on growth and development of infant rats.

Method. Over 200 newborn albino rats of mixed strain were used. Wherever possible, the individual litters were divided so that there were 4 or 5 rats receiving the test drug and an equal number of litter-mates of the same sex distribution acting as controls. Unless otherwise stated, two or more litters were used for each experimental dose. After marking the rats for identification, the experimental and control rats were returned to the mother so that environmental and nursing conditions were identical. Injections using non-sterile technic were given subcutaneously and were usually begun when the rats were approximately 24 hours old; their weight at that time was 6 or 7 g. The control rats received injections of the solvent or suspending medium of the experimental drug. Observations were made once or twice daily on body weight, eruption of incisor teeth, development of the gingivae, and opening of the eyelids.

Results. Adrenocorticotropic hormone (ACTH) was injected in doses of 0.1 mg to 0.75 mg (of LA1-A Standard) per day usually in 2 or 3 divided doses for 8-12 days. With doses up to 0.4 mg per day, little or no effect was noted on body weight or eyelid and tooth development. When the dose was increased to 0.75 mg per day, a 15-20% decrease in body weight as compared to litter-mate controls was noted after 7 days. Upon cessation of injections, there occurred a rapid gain in weight so that by the 14th to 18th day of life, the weight was equal to that of the controls. There was only a questionable effect on development with very slight effect on eye opening or tooth eruption. Higher dose experiments are planned now that the supply of ACTH is greater.

Corticosterone was injected into 5 rats in single daily doses of 0.2 mg for 8 days. No demonstrable effect was noted on body weight but a definite hastening of teeth eruption and eye opening occurred. The experimental animals had erupted incisor teeth and showed opening of the eyelids 1 to 1½ days before their controls.

Cortisone. A single injection of 0.25 mg of cortisone at 24 hours of age resulted in a failure to gain weight normally for several days. By two weeks of age, the rats had recovered sufficiently to approach the weight of their litter-mate controls. Two consecutive daily injections of 0.25 mg beginning on the second day of life resulted in a similar effect on body weight but by the second week, they still weighed several grams less than their controls. Three or more consecutive daily injections of 0.2 mg beginning at 24 hours of age resulted in death of most of the animals. Apparently 0.6 mg or over of cortisone given in the first few days of life is a lethal dose.

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* New York City. ACTH, Armour Lab.; Cortisone, Merck & Co.; Corticosterone, courtesy of Dr. E. C. Kendall; DCA and Pregnenolone, Schering Corp.

1. Mulinos, M. G., and Parmer, L. G., *Science*, 1942, v95, 484.

2. Parmer, L. G., Proc. Soc. Exp. Biol. and Med., 1947, v66, 574.

This indicates that the baby rat is markedly sensitive to cortisone as compared with other adrenal hormones studied. Five daily injections of 0.1 mg of cortisone beginning at 24 hours of age resulted in marked inhibition of growth with stimulation of development as judged by eruption of teeth, separation of the lips from the dental gingiva, and opening of eyelids. The teeth erupted on the average of 2.5 days sooner and the eyes 2.8 days sooner than litter-mate controls. A similar dose begun after 48 hours of age had slightly less effect on growth inhibition as well as developmental changes (1.6 days difference in teeth eruption and 1.8 days for eye opening). Injection of 0.15 mg of cortisone for 5 days produced results similar to the 0.1 mg dose except that body weight gain was slightly more retarded. With either dose, the inhibition of body growth and the failure to regain weight equal to that of the controls persisted for the duration of the experiment (90 days) at which time some of the cortisone-treated rats weighed 10 to 15% less than the controls.

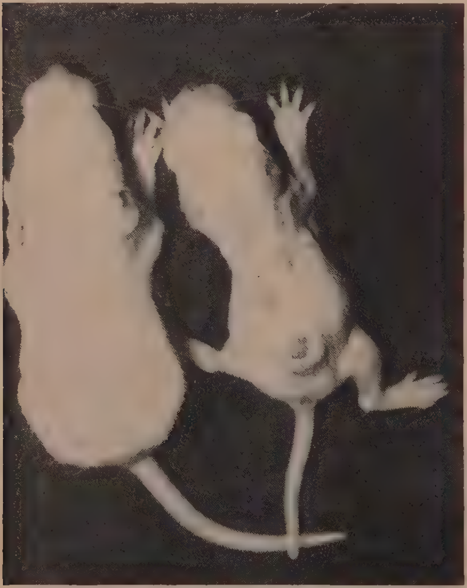

FIG. 1. Effect of cortisone on growth. Rats are litter-mate males, age 16 days. The one on right weighing 15 g received subcutan. over back area 0.1 mg cortisone/day for 5 days during first week of life. Note incidental infection at injection site. The other rat weighing 23 g received corresponding volume of suspending medium as control injection.

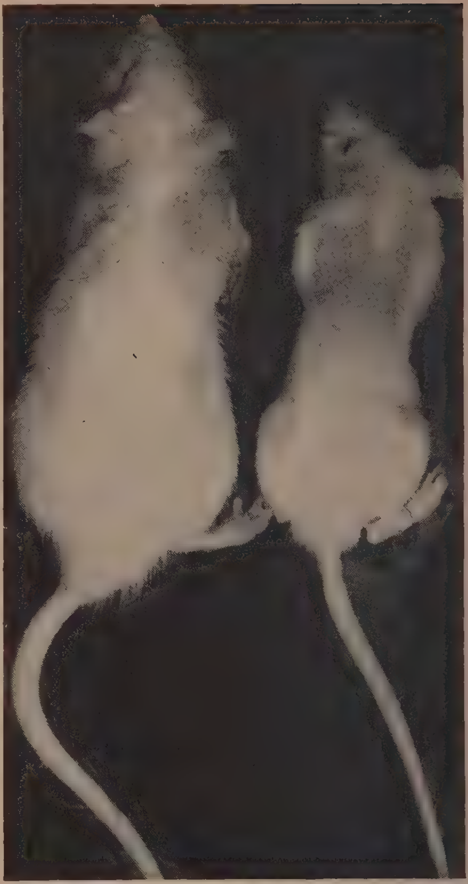

FIG. 2. The same rats 6 weeks later. This cortisone-treated rat which was the most severely stunted in its group weighed half as much as its litter-mate control.

It was noted that many of the cortisone-treated rats were more susceptible to infection as judged by the occurrence of subcutaneous infections at the injection site and the development of diarrhea with loose yellow stools. It was not clear whether or not this was nonspecific and related to their general ill health. Hair growth was also found to be somewhat retarded in cortisone-treated rats. As an incidental finding, it was observed that 0.1 mg of cortisone per day for 3 days hastened the death of infant rats that were malnourished. This occurred in one litter where the mother became temporarily ill, and could not nurse well. All 5 of the experimental rats died on the average of 2 days before the controls began to succumb. Three controls died and 2 survived. It would appear that this particular dose of cortisone is detrimental to infant rats during a period of stress due to malnutrition.

Toxicity to cortisone in the infant rat is mainly demonstrated by failure to gain weight. (Fig. 1, 2). At no time was edema seen such as is reported for man(3). On the contrary, the higher the dose, the more dehydrated the animal became. This may have been a reflection of a failure to nurse well plus the fact that a nursing rat is on a relatively low sodium intake.

Desoxycorticosterone was injected in doses of 0.25 mg per day for 10-14 days. The results of a previous report were confirmed(1). Even with a higher dose of 0.5 mg per day, no marked effect on the growth curve was elicited in most cases. Eruption of incisor teeth and opening of eyelids occurred about 1 to 1½ days before controls.

Pregnenolone was given to one group of rats in a dose of 5 mg per day for 13 days. Although a total dose of 65 mg was injected, no discernible effect was noted on body weight, teeth eruption or eye opening. No evidence of toxicity was apparent. At autopsy at 18 days of age, there was a considerable amount of unabsorbed drug at the injection site but without signs of inflammation.

Discussion. The infant rat lends itself readily for studies on growth and development. Adequate litter-mate controls on an identical dietary and environmental status are available. The animal is a rapidly growing one and its steep growth curve can act as a sensitive measure of factors affecting body weight. The newborn rat is markedly undeveloped with no teeth or hair, sealed eyelids and fusion of the lips to the gingivae. These externally visible characteristics serve as an indicator of at least one phase of development. Normally, all animals in a litter develop at about the same rate. Therefore, differences can readily be detected by comparing treated animals to their untreated litter-mates. Another advantage of infant rats is that both males and females grow at the same rate for the first few weeks of life. If older animals are used for growth experiments, scrupulous attention must be paid to the sexes because the male rat rapidly outgrows the female. Finally, with scarce hormones and drugs, less material is needed for study when infant rats are employed.

The effect of cortisone on growth has been known for many years. Wells and Kendall(4) reported that feeding of 1 mg of cortisone daily to rats weighing 30 to 40 g resulted in marked suppression of growth. They weighed about 30 g less than their controls at the end of only 10 days of treatment. A similar experiment with corticosterone resulted in growth inhibition to a much lesser degree. Recently, Winter *et al.*(5) injected daily doses of 3 mg of cortisone into rats weighing 200 g with complete cessation of growth during the 40-day injection period. Fifty days later, although their growth had resumed, the rats were still markedly underweight. It is of interest that the cortisone-treated animals consumed much more food than control animals whose food intake was restricted in order to maintain the same body weight as the injected animals. Li has reported that ACTH also retards body growth probably by means of increasing nitrogen excretion(6). When rats were hypophysectomized and injected with both ACTH and growth hormone, the effect of each compound was nullified by the action of the other.

The present experiment extends the observations on older rats to newborn rats. Both cortisone and ACTH are potent growth inhibitors. However, on a strictly drug weight basis, cortisone is the more effective growth inhibitor for the infant rat. Table I shows the lack of correlation between growth inhibition and precocious eruption of teeth and opening of eyelids by means of various sub-

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3. Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F., *Proc. Staff Meet.*, Mayo Clinic, 1949, v24, 181.

4. Wells, B. B., and Kendall, E. C., *Proc. Staff Meet., Mayo Clinic,* 1940, v15, 324.

5. Winter, C. A., Silber, R. H., and Stoerk, H. C., *Endocrinology,* 1950, v47, 60.

6. Li, H. C., *Growth Symposium,* 1948, v12, 47.

TABLE I. Comparative Effects of Various Substances on Infant Rats. Injections Begun at One Day of Age.

Substance	Daily dose	Days treated	Total dose	Retardation of growth	Hastening of tooth eruption and eye opening
Cortisone	.1 mg	5	.5 mg	4+	4+
Aqueous adrenal extr.(1)	.2 ml	10	2 ml	0	3+
Desoxycorticosterone	.25 mg	14	3.5 mg	0	3+
Corticosterone	.2 ”	8	1.6 ”	0	2+
ACTH	.75 ”	8	6 ”	2+	±
Pregnenolone	5. ”	13	65 ”	0	0

stances. It also reflects the fact that the infant rat can withstand much larger doses of DCA than cortisone. This is the reverse of what occurs in man where relatively small doses of DCA are toxic as compared with the dose of cortisone. The dose of DCA that can be administered to man is limited by virtue of its marked effect on electrolyte and water balance. Apparently, this sign of toxicity is not manifested in the infant rat. The present experiments do not shed light on the mechanism of the precocious changes. Both the adrenal hormones affecting carbohydrate metabolism and those affecting electrolyte metabolism produce these changes. It does not appear to be due to a non-specific effect of the steroids because pregnenolone which structurally resembles the other substances is ineffective.

Summary. 1. ACTH, cortisone, corticosterone, desoxycorticosterone and pregnenolone were injected into newborn rats in varying dosage. Cortisone was found to be the most toxic with a total of 0.6 mg in the first few days of life being a lethal dose, as contrasted with the lack of toxicity of 0.25 mg of desoxycorticosterone given daily for 14 days. Cortisone was the most potent growth inhibitor and also the most potent stimulus for eruption of teeth, opening of eyelids and development of the gingivae. ACTH, although employed in larger doses than cortisone had a lesser effect on growth inhibition and little or no effect on development. Larger doses of ACTH are yet to be used. Corticosterone in the dose employed did not affect growth but did stimulate development. Pregnenolone in huge doses had no effect on either growth or development of teeth and eyelids. 2. Cortisone in a total dose of 0.5 mg (sub-lethal) given to newborn rats in the first week of life resulted in long-term damage to the animal as evidenced by failure to regain normal body weight 3 months after cessation of injections. The younger the animal at the time of the first injection, the more marked was the growth failure.

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Content of Radioactive Vitamin B_{12} in the Feces of Rats Fed Co^{60} and Aureomycin.* (18729)

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The importance of trace elements such as cobalt for optimal growth and normal health was long recognized by McCollum(1). Gall (2) demonstrated that the oral administration

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* This work was supported by grant-in-aid from Sharp and Dohme, Merck and Co., and Upjohn and Co.

1. McCollum, E. V., Orent-Keiles, E., and Day, H. G., *The Newer Knowledge of Nutrition,* New York, 1944, The Macmillan Co.

of "cobalt" resulted in stimulation of certain bacterial organisms in the rumen of sheep. In a similar experiment Abelson and Darby (3) fed radioactive cobalt (Co^{56}) and isolated from feces a fraction soluble in n-butanol and containing radioactive Co^{56}. This fraction was found, also, to possess the microbiological activity of vit. B_{12} as measured by *L. leichmannii*. These reports, therefore, indicate that at least a part of cobalt in the diet is utilized for the synthesis of vit. B_{12} molecule, presumably by the organisms in the intestinal flora. Stokstad(4) and his associates found that growth responses of chicks on a corn-soybean diet adequately supplied with vit. B_{12} were stimulated as a result of supplementation with crystalline aureomycin and less effectively by other bactericidal agents such as succinyl-sulfathiazole, streptomycin, and 3-nitro-4-hydroxy-phenyl arsonic acid. Similar results from experiments using swine were reported by Edwards *et al.*(5) and Jukes *et al.*(6). On the other hand, studies by Colby *et al.*(7) demonstrated that feeding aureomycin to lambs produced harmful effects, such as decrease in feed consumption and loss in body weight. They attributed their observations to the inhibition of certain strains of bacteria in the intestinal flora by aureomycin.

The above data demonstrate that the addition of aureomycin in excessive amounts may result in deleterious effects, whereas supplementation in appropriate amounts may stimulate growth response, possibly through alteration of the flora so as to result in an increase in the synthesis of certain vitamins utilizable by the host. Direct experimental evidence for the latter effect, however, is still lacking. Hence, it would be of interest to demonstrate, if possible, an increase in the synthesis of vitamins by comparing the contents of a certain selected vitamin in the fecal matter of rats fed a diet with or without supplementation with aureomycin. Since the vit. B_{12} molecule contains an atom of cobalt which can be isotopically tagged, the synthesis of the vitamin by the micro-organisms in the intestines can be readily followed. This communication reports the results of such a study.

Experimental procedure. The composition of the control soybean diet is: 62% soybean meal, 29.5% sucrose, 4% salts mixture IV, and 4.5% corn oil. The vitamin supplement for each kg diet consists of 2.0 mg thiamine, 3.0 mg riboflavin, 2.5 mg pyridoxine, 20.0 mg calcium pantothenate, 50 mg niacin, 100 mg inositol, 0.1 mg biotin, 250 mg p-aminobenzoic acid, 0.2 mg folic acid and 1 g choline HCl, 210 mg percomorph oil, 23 mg vit. E, 2.1 mg vit. K, 12,600 units vit. A, and 1,785 units vit. D. Approximately year-old male and female rats were used for the experiment. They were raised from the time of birth on the control soybean diet deficient of vit. B_{12}. At the beginning of the experiments the males weighed approximately 380 g and the females 300 g. They were kept in individual metabolism cages and were acclimated to the control diet, with or without aureomycin supplement for a period of 7 days. Their dietary intake was limited to 12 g per rat per day during the entire period of study. On the eighth day, one ml of $CoCl_2$ solution containing 0.2 to 0.4 μg of radioactive cobalt $\cong$ 20,000 to 40,000 c.p.m. was measured into every portion of the daily dietary allowance of rats in both the control and aureomycin groups (Table I). Urine specimens were discarded. Feces were collected every 48 hours. They were weighed and homogenized with about 80 ml of cold distilled water, and the total volume was recorded. After centrifugation in the cold, a 10 ml aliquot of the clear homogenate was removed, and an equal volume of saturated ammonium sulfate was added to it. The solution was extracted 4 times with equal volumes of normal butanol.

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3. Abelson, Philip H., and Darby, Hugh H., *Science*, 1949, v110, 566.

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5. Edwards, H. M., Cunha, T. J., Meadows, G. W., Sewell, R. F., and Shawver, C. B., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 445.

6. Jukes, T. H., Stokstad, E. L. R., Taylor, R. R., Cunha, T. J., Edwards, H. M., and Meadows, G. W., *Arch. Biochem.*, 1950, v26, 324.

7. Colby, R. W., Rau, F. A., and Dunn, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 234.

TABLE I. Radioactivity* of the Butanol Extracts of Feces of Rats on Various Test Diets.

Group	No. of rats	% aureomycin in diets	μg† Co^{60} added/day	Assay period (48 hr)				
				1st	2nd	3rd	4th	5th
W	6	.0	.2	1.3	1.5	1.5	1.7	1.6
X	6	.04	.2	3.1	6.1	5.1	5.1	5.7
Y	3	.1	.2	4.4	5.3	6.0	7.0	5.6
U	3	.1	.4	8.2	10.2	9.5	10.8	8.8
Z	3	.0	.4	3.3	—	—	—	—

* Avg of group in c.p.m. × 10^{-3}.
† One $\mu g \Bumpeq 100{,}000$ c.p.m.

The butanol extracts were pooled and centrifuged to remove particulate matter of the aqueous phase that might have been carried over mechanically. A small aliquot (0.5 ml) of supernatant was saved for microbiological assay which was not affected by the amount of butanol present after the necessary dilution of some 100-fold. The remaining portion of butanol extract (approximately 80 ml) was boiled off until the volume was reduced to approximately 2-3 ml, the concentrate was transferred quantitatively to a counting pan. The contents were dried, and the radioactivity of the solid was measured with Geiger-Müller instrument.

The data in Table I indicate that the amount of radioactivity of the cobalt compound, soluble in the butanol extract of fecal matter in any single group of rats, was essentially constant over the 5 periods of observation. It can be noted that the addition of aureomycin to the diet brought about a several-fold increase in the radioactivity (compare Groups W and X). It appears that supplementation with 0.04% aureomycin produced a maximum effect since a higher level of the antibiotic did not bring about systematically any added increase in radioactivity (compare Groups X and Y). However, when the daily cobalt supplement was raised to 0.4 μg per rat the radioactivity was increased (compare Groups U and Y). Animals in Group Z received the same amount of cobalt chloride but no antibiotics. Although we have data only for the first period, they indicate that the addition of $cobalt^{60}$ may be a limiting factor in the biosynthesis of the organic Co-complex in the intestinal flora. This is in harmony with the results of Hendlin and Ruger(8) who studied, *in vitro*, the cobalt requirements of *S. griseus*.

Even though results in Table I demonstrate the increase in the radioactivity in the butanol extract following supplementation of aureomycin, this effect might be accounted for if our procedure does not quantitatively free the organic phase from the inorganic cobalt salt. To eliminate such a possibility, we have added 0.4 μg $cobalt^{60} \Bumpeq 40{,}000$ c.p.m. and 313 $m\mu g$ of vit. $B_{12} \Bumpeq 3130$ c.p.m. to a fecal sample from a rat previously offered the control diet free from any radioactive substance. The recovery in the butanol phase, by analyses of radioactivity, was 2,400 c.p.m. or 75% in terms of radioactive B_{12} added and by microbiological analysis was 250 $m\mu g$ or 80% of vit. B_{12} added. These data, therefore, show satisfactory separation of vit. B_{12} from $CoCl_2$ under our experimental conditions. Since the cobalt-containing compound soluble in the butanol phase is essentially free from the cobalt ion, it is conceivable that cobalt might be coordinated with some organic molecules to form compounds soluble in normal butyl alcohol. Such compounds will possess radioactivity of $cobalt^{60}$ but need not have the microbiological activity of vit. B_{12}.

It is, therefore, important to correlate radioactivity with microbiological activity of the fraction soluble in butanol. The data collected in Table II demonstrate that Group W, which has the lowest count, has the least microbiological activity, whereas Group U is highest in both. The ratios of radioactivity to microbiological activity of the fecal samples from different groups of rats are essentially constant (see last column). According to this constant, there were about 2 x 10^{-3} c.p.m. per μg of vit. B_{12} found by microbiological assay. The results, therefore, suggest that the butanol phase contains vit. B_{12} or B_{12}-like substance. Additional evidence may be obtained by correlating other physical characteristics of vit. B_{12} with the radioactivity of Co^{60}. However, final proof could be

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8. Hendlin, D. and Ruger, Myrle L., *Science*, 1950, v111, 541.

TABLE II. Correlation of Microbiological and Radioactivity in the Butanol Extract.

Group*	No. of rats	A = Radio-activity avg of group c.p.m. × 10-3 per sample	B = Micro-biological activity group avg expressed in γ/sample B_{12}	A/B
W	6	2	.9	2.2
X	3	6	2.8	2.1
Y	2	5	2.0	2.5
U	3	9	3.4	2.6

* See Table I for composition of group diets.

TABLE III. Fecal Weights of Rats on Diets with or Without Aureomycin.

Group*	No. of fecal samples	Sex	Avg wt of feces in g/2-day collection
W	26	F	3.1 ± .23
X	26	F	4.2 ± .32
Y	6	M	5.3 ± .3
U	11	M	6.3 ± .4

* See Table I for composition of group diets.

reached only by actual isolation.

Another observation which is worth recording deals with the relative fecal matters collected from different groups of rats. The fecal weights are tabulated in Table III. Although all animals had approximately the same diet allowance, the feces of those in the aureomycin groups (X, Y, and U) weighed more than those in the control group W. Furthermore, this difference in the fecal weight manifested itself not only during any single period but was sustained during the entire experiment.

Discussion. The fact that the addition of antibiotics in appropriate amounts can improve the nutritional status of the host can be accounted for on the assumption that the bactericidal agent selectively destroys certain microorganisms, (a) which will otherwise compete with the host for accessory factors synthesized by another group of organisms, or (b) which produce toxins that might interfere with the utilization of nutrients. The data presented in this paper provide direct evidence that the addition of aureomycin results in an increase in the vit. B_{12} content of the fecal matter. Our experiments do not exclude the possibility that the fecal content of other accessory factors might also have been increased as a result of aureomycin feeding. Such an hypothesis could explain some of the findings of Jukes *et al.*, who demonstrated some beneficial effects to growing animals after supplementation of aureomycin in diets with adequate supply of vit. B_{12}. It is also important to point out that, in this series of experiments, only aureomycin was used, although other antibacterial agents might be expected to produce similar effects.

Summary. By the incorporation of Co^{60} into the diet, it could be shown that aureomycin causes an increase in the activity, both radioactive and microbiological, of the vit. B_{12} content of the feces. The fecal B_{12} content could be increased by increasing the amount of Co^{60} used. The experimental findings can be explained by the effect of the antibiotic on the intestinal bacterial flora.

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Filtrates of Fresh Tumor Injected Prior to Transplantation of the Homologous Tumor.* (18730)

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Anaerobic refrigeration in the deep freeze (0-24°F) for 10 days or longer (until the cells are no longer viable) or lyophilization for a similar period of Brown-Pearce tumor tissue(1,2), Bashford Ca 63(3-5), 15091a (6-8) or Eo771(6-9) tumor tissue results in the presence of specific(5-9) substances which,

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TABLE I. Preparation of and Injection Intervals of Filtrates of Fresh Untreated Emulsions of the Brown-Pearce Tumor.

Exp.	Tumor type	Filtrate site inoc.	Tumor int. days	Transplant site inoc.	Experimental				Controls			
					N	I	D	T	N	I	D	T
1	BVF	LT	14	RT	5	1	0	6	1	3	2	6
2	BVF	LT	14	RT	0	1	3	4*	2	2	2	6
3	BNF	SC	13	SC	3	2	0	5	4	1	0	5
4	SF	SC	20	SC	3	1	1	5*	3	0	1	4*
5	SF	SC	23	SC	4	1	0	5	1	0	2	3*
6	SF	SC	21	SC	3	1	1	5	1	2	1	4*
				Totals	18	7	5	30	12	8	8	28

Exp.—experiment No.; BVF—Berkefeld "V" filtrate; BNF—Berkefeld "N" filtrate; SF—Seitz filtrate; LT—left testis; RT—right testis; SC—subcutaneous; int. days—time interval between inoculation of filtrate and tumor transplantation; N—animals without primary or metastatic tumor at necropsy; I—animals alive but with primary or metastatic tumor at necropsy; D—animals dying from growth and metastases of the Brown-Pearce tumor; *—animals dying from intercurrent disease under 21 days after tumor transplantation were omitted from the tabulation (Table II).

when injected into animals prior to transplantation of the homologous tumor, cause the transplantation to be more successful in homologous(9) and in heterologous(1,6-8) hosts, and the metastases and mortality to be increased(1,2). The Brown-Pearce "autolyzed" material (called XYZ) was filtrable through a Berkefeld "V" filter(10).

Attempts were then made to see whether the same effect could be induced by injecting a cell-free filtrate of fresh tumor tissue 10-14 days before tumor transplantation. Berkefeld "V"(11) and Seitz(12,13) filtrates of fresh Brown-Pearce tumor failed to induce a state of susceptibility in the injected animals. In fact, the reverse effect was observed. Since marked physical and perhaps chemical disintegration of the cells was induced by the repeated freezing and thawing before filtration employed by Raab and by Malluche(12,13), it seemed worthwhile to do additional experiments with the Brown-Pearce tumor using filtrates of fresh tumor tissue which had not been killed and disintegrated by rapid freezing and thawing.

Materials and methods. In 6 experiments 66 rabbits were injected intratesticularly or subcutaneously with an emulsion of Brown-Pearce tumor tissue. The methods of preparation, inoculation, postmortem examination and analysis have been described(1,2). There were 33 control and 33 experimental animals matched as closely as possible as to age, breed, weight, etc. (Table I). The animals were chosen on receipt from dealer on the basis of apparent health yet 6 experimental and 5 control animals died of intercurrent affection during the 3 to 5 months which the experiments lasted, 8 of these shortly after reception. No further disease was noted at postmortem examination. In 2 experiments 0.3 cc of a Berkefeld "V" filtrate of fresh tumor tissue (1-4 emulsion in sterile pneumococcus broth pH 7.8) was injected into the left testis and 14 days later 0.3 cc of a saline emulsion of Brown-Pearce tumor transplanted into the right testis. In a third experiment a Berkefeld "N" filtrate of fresh Brown-

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1. Casey, Albert E., PROC. SOC. EXP. BIOL. AND MED. 1932, v29, 816; *Am. J. Cancer*, 1934, v34, 760.
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Pearce tumor was injected subcutaneously 13 days before subcutaneous transplantation of the tumor. In 3 additional experiments 0.3 cc of a Seitz filtrate of fresh tumor tissue (Brown-Pearce) was injected once 20-23 days prior to subcutaneous transplantation of the Brown-Pearce tumor.

Results. The results are summarized in Table II. The percentage of deaths, the average number of metastatic foci and the total tumor per animal were less (statistically insignificant) and the number of animals which failed to grow the tumor more (statistically insignificant) among the groups of animals having pre-transplantation treatment with the filtrates. It was concluded therefore that the XYZ factor for the Brown-Pearce tumor either was not present or was biologically inactive in filtrates (Berkefeld V, N and Seitz) of fresh emulsion of the Brown-Pearce tumor.

Discussion. Domagk and Hackmann(14) and Dittmar(15) injected filtrates of fresh Ehrlich mouse carcinoma or sarcoma prior to tumor transplantation, and recorded inhibitory results similar to ours with the Brown-Pearce tumor. However, no XYZ factor has been demonstrated for this tumor. Recently Kaliss, Jonas and Avnet(16) reported in an experiment employing 21 animals that a filtrate of fresh mouse mammary tumor 15091a failed to sensitize a heterologous strain of mice to the homologous (15091a) tumor, whereas the lyophilized tumor tissue was quite potent (6-8) in this respect.[†]

The complete experience of Raab, Malluche and ourselves with the Brown-Pearce tumor, of Dittmar with the Ehrlich mouse sarcoma and of Kaliss, Jonas, and Avnet with 15091a was summarized (Table III). In each experiment by each of the authors the fresh filtrate-injected animals grew fewer tumors than the controls (not statistically significant). This is in contrast to the statistically significant and widespread tumor growth and metastases following a preliminary injection of a Berkefeld "V" filtrate of homologous tumor tissue which has been kept frozen (the tumor tissue) for 10-21 days(10). Since the Brown-Pearce and other XYZ or lyophilization factors seem specific and always present in the homologous refrigerated tumor tissue they must be "bound" or in some combination in fresh tumor,[‡] and must be activated

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14. Domagk, G. and Hackmann, Ch., *Z. f. Krebsforsch,* 1935, v42, 192.

15. Dittmar, Carl, *Z. f. Krebsforsch,* 1936, v45, 109.

16. Kaliss, Nathan, Jonas, Gordon and Avnet, Norman L., *Cancer Research,* 1950, v10, 228.

[†] The specific lyophilization or XYZ factor of mouse carcinoma 15091a(6,7,8,9) was present but in less active form in fresh(16) unfiltered tumor tissue and in its sedimented cells (28 of 63 C57 mice or 44% grew this Line A tumor; 0% of the controls) and was abundant in lyophilized(8) tumor tissue (141 of 184 C57 mice or 77% grew the Line A tumor; 0% of 202 controls). The factor was not demonstrated in the filtrates of fresh tumor (0 of 21 or 0%) but was suggestively present in fresh unfiltered normal liver, spleen and kidney of Line A mice (13 of 82 C57 mice or 16% grew the Line A tumor; 0% of controls) and definitely present in lyophilized normal liver, spleen and kidney of Line A mice (47 of 105 C57 mice, or 45%, grew the Line A tumor; 0% of controls). The question arises as to whether the specific 15091a factor is a silent virus in the sense of the "milk agent" widely distributed in an inbred line of mice(17), or a somatic host factor similarly placed. The specific XYZ factor in C57 black mammary carcinoma E0771 did not seem to be a somatic host factor since it could not be demonstrated in C57 black mouse mammary carcinomata 241-5, 241-16, 755, or 946 or in several other neoplasia of C57 black origin (9,18). Furthermore, the XYZ phenomenon seems to be a technic by means of which the E0771 XYZ factor can be readily eliminated from this tumor(18) and the specific factor, therefore, is not somatic.

17. Barnum, Cyrus P., Ball, Z. B., and Bittner, John J., *Cancer Research,* 1947, v7, 522.

18. Casey, Albert E., *Acta, Fifth International Cancer Congress,* Paris, France, July 17, 1950; Annual Rep. to Com. on Growth, Nat. Research Council, acting for the American Cancer Society.

[‡] However, an accelerating factor was demonstrated in the unfiltered supernatant fluid which had been prepared by centrifugation repeatedly of a saline extract of fresh (unfrozen) mouse mammary carcinoma Z8352. This tumor, which originated in a female C3H mouse possessing the milk agent, is maintained by transplantation in ZBC mice not possessing the milk agent(22).

22. Shear, Howard H., in press.

TABLE II. Results of the Prior Injection of Filtrates of Fresh Tumor on the Course of the Brown-Pearce Rabbit Tumor.

A. Average number of metastases and average volume of primary and metastatic tumor.

	Per animal inoculated						Per animal with tumor					
	Filtrates			Controls			Filtrates			Controls		
Sites	R, #	MF, #	TT, cc	R, #	MF, #	TT, cc	R, #	MF, #	TT, cc	R, #	MF, #	TT cc
LT–RT	10	6.2	34	12	6.8	52	5	12.4	68	9	9.1	70
SC–SC	20	1.8	30	16	3.9	64	7	5.0	84	7	8.9	146
Total	30	3.2	31	28	5.2	59	12	8.8	78	16	9.1	103

B. Animals dying from tumor growth versus animals completely negative.

	Negative				Died from tumor				Alive with tumor			Total	
	Filtrates		Controls		Filtrates		Controls		Filtrates		Controls		
Sites	#	%	#	%	#	%	#	%	#	%	#	F	C
LT–RT	5	50	3	25	3	30	4	33	2		5	10	12
SC–SC	13	65	9	56	2	10	4	25	5		3	20	16
Total	18	60	12	43	5	17	8	29	7		8	30	28

R—rabbits; MF—avg No. of metastatic foci per animal; TT—avg volume of primary and metastatic tumor per animal; filtrates—animals treated with filtrates of fresh Brown-Pearce tumor 13-23 days before transplantation of the Brown-Pearce tumor; controls—animals not so treated; #—No.; cc—cubic centimeters, measured by water displacement; LT—left testis, site of filtrate injection; RT—right testis, site of tumor transplantation; F—filtrates; c—controls; A.W.T.—indeterminate (alive with tumor at the termination of the experiment); none of the differences between filtrate and control series were statistically significant.

TABLE III. Summary of Results by Various Authors of the Injection of Filtrates of Fresh Tumor Prior to Tumor Transplantation.

		Animals			Negative				Grew tumor			
					Filtrate		Controls		Filtrate		Controls	
Author	Tumor	F #	C #	Total #	#	%	#	%	#	%	#	%
1 LT–RT	BP	10	12	22	5	50	3	25	5	50	9	75
1 SC–SC	BP	20	16	36	13	65	9	56	7	35	7	44
2 IT–IT	BP	46	120	184	16	35	34	28	30	65	86	72
2 SC–IT	BP	18			7	39			11	61		
3 IV–IT	BP	29	12	41	3	10	1	8	26	90	11	92
Subtotal	BP	123	160	283	44	36	47	29	79	64	113	71
4 SC–SC	EMS	159	102	261	43	27	17	17	116	73	85	83
5 SC–SC	15091a	11	10	21	11	100	10	100	0	0	0	0
Total		293	273	565	98	33	74	27	195	67	198	73

F—filtrate-injected animals; C—control animals; Total—total animals; 1—see Table II; 2—Malluche(13); 3—Raab(12); 4—Dittmar(15); 5—Kaliss, Jonas and Avnet(16); LT-RT—filtrate injected into left and tumor into right testis; SC—subcutaneously; IT—intratesticularly; IV—intravenously; BP—Brown-Pearce rabbit tumor; EMS—Ehrlich mouse sarcoma; 15091a—mammary carcinoma 15091a of Line A origin.

by slow autolysis in the cold, or are attached to or parts of a macromolecule which is not filtrable through Berkefeld or Seitz filters. The slight inhibitory influence noted in filtrates of fresh tumor tissue could be of the nature described by Gorer(19), by Kaliss and Jay(20), and by Murphy, Helmer, Claude and Sturm(21) and others. It would seem to bear no relation to the specific XYZ or lyophilization factors found in Brown-Pearce, Bashford 63, Eo771 or 15091a tumors.

Summary. In 6 experiments involving 66 rabbits, Berkefeld "V", "N" and Seitz filtrates of fresh Brown-Pearce tumor tissue did not contain evidence of the specific XYZ or lyophilization factor, although the factor has been uniformly present in Berkefeld "V" filtrates of tumor tissue after prolonged freezing.

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21. Murphy, J. P., Helmer, O. M., Claude, A., and Sturm, E., *Science*, 1931, v73, 268.

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Intracellular Localization of Compounds Labeled with Tritium, H^3, By Radioautography.* (18731)

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(Introduced by C. P. Rhoads.)

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The ultimate goal of radioautography is the localization of an element or compound to small intercellular areas or to intracellular structures. The demonstration of radioactivity in human or animal organs or parts of an organ has often been achieved(1) and the concentration of an isotope in individual isolated cells demonstrated(2). Attempts at finer localization have been limited by two factors—lack of emulsion resolution and the long average path in emulsion of many isotopes. Resolution of emulsions has been recently increased by the nuclear track emulsions so that lines of 2.5 μ width have been resolved(3). This suggests the possibility of intracellular localization. However, the maximum paths in the nuclear emulsions of even such weak beta particle emitters as $carbon^{14}$ and $sulfur^{35}$ are approximately 90 and 100 μ, respectively. Under-exposure and long development of emulsions considerably reduce these disadvantages by making visible individual beta particle tracks(4). The initial high energy of beta emitters causes wide grain spacing at the beginning of the track and this detracts from efforts towards the microscopic localization of a radioactive particle to its site of emission. Alpha particle emitters are foreign to most biological organisms and no extensive use of them as a label has been reported.

We have employed tritium, H^3, whose average beta particle traverses less than 1 μ in nuclear track emulsions, to give radioautographic images of individual yeast cells and intracellular paramecium structures (Fig. 1, 2).

Experimental. *Paramecium aurelia* were grown for 7 days in a Timothy hay infusion medium in which *Aerobacter aerogenes* had been incubated at 27°C for a 48-hour prior period. The medium included aqueous solution of sodium acetate containing tritium. The activity of the water was 2×10^7 disintegrations per min. per mg and the sodium acetate, labeled in the methyl groups with tritium, had an activity of 4×10^7 disintegration per min. per mg. After the growth period paramecia were fixed in 2% aqueous osmic acid solution. The mixture was centrifuged and the organisms washed with distilled water and fixed in 2% of U.S.P. formalin solution for 2½ hours. After being washed in distilled water and dehydrated in 70, 95, and 100% alcohol, the organisms were placed in an ether-alcohol solution, embedded over a period of 2 days in celloidin and finally in 2 changes of paraffin to give a rigidly embedded block. Sections were cut at 1 μ on a wedge type microtome(5). Whole organisms were put through the same initial procedures but after being fixed, were washed, centrifuged, and kept in distilled water. A control group of organisms was processed simultaneously under similar circumstances with the exception that the medium contained no radioactivity.

Torula utilis, a saprophytic yeast, was grown in a salt solution(6) in which the only source of nitrogen was ammonium ion and the

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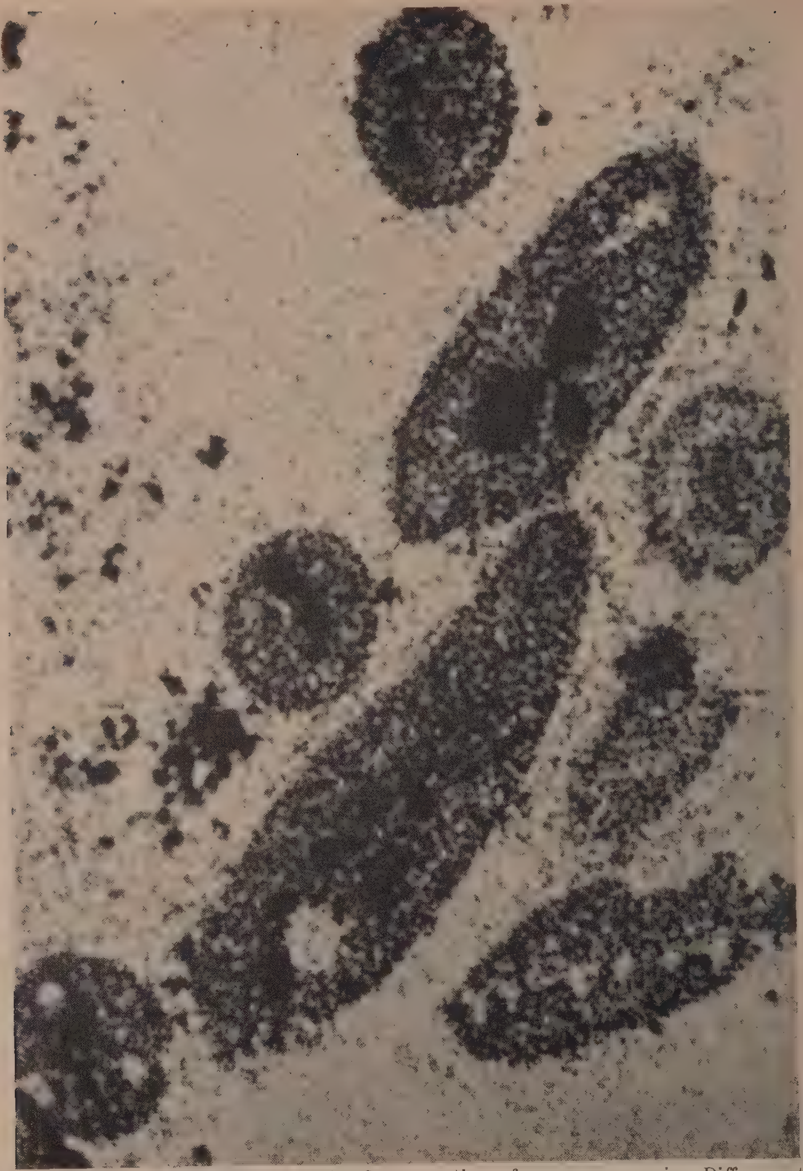

FIG. 1. Radioautograph (#51-24-K). One μ section of many paramecia. Diffuse activity with endoplasmic concentrations. Some areas of activity between organism (radioactive organisms or debris). Exposure two weeks, E.K. NTB_3, 10 μ, emulsion. Unstained. Original magnification 460.

only source of carbon was sodium acetate present in a concentration of 2%. The test organisms were grown in 2 ml of medium in a shaken culture for 4 days at 25°C. The activity of the sodium acetate was the same as used for the paramecium but the activity of the water was 2×10^6 disintegrations per min. per mg. After growth in the medium

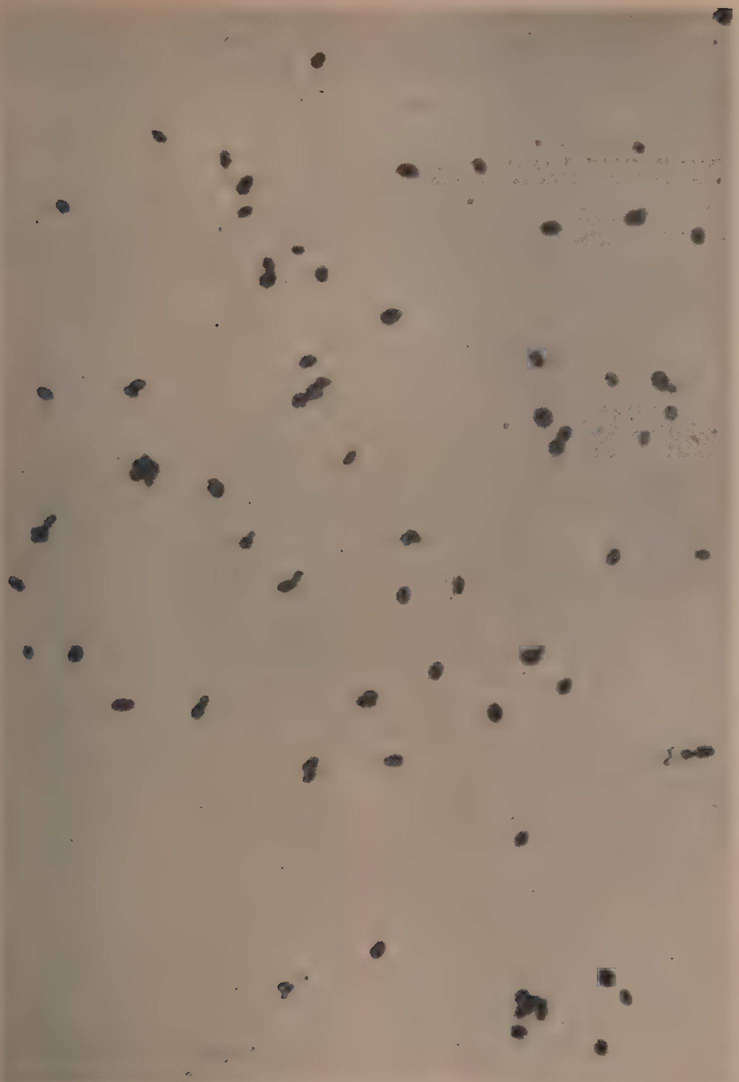

FIG. 2. Radioautograph (#51-35) of yeast cells obtained by placing Kodak Limited NTB stripping film over smear of dried organisms. Three weeks exposure. Unstained. Original magnification 492.

containing tritium, the yeast were washed with distilled water and centrifuged 6 times and kept in distilled water. Control organisms were simultaneously processed with the exception that the medium contained no radioactivity.

Radioautographic technic. Only the nuclear track types of emulsion were used. Eastman Kodak NTB, NTB_2, NTB_3 emulsions and a Kodak Limited stripping film from autoradiographic plates were used. A drop of distilled water containing the radioactive yeast or paramecia was placed on these emulsions. Drops of organisms were placed on glass slides, dried, and the slides were apposed to the emulsion surfaces. The stripping film was placed over dried organisms on the slide. Sections of the paraffin-celloidin embedded organisms were transferred to a water bath, floated on to photographic emulsions, deparaffinized, dried and exposed. Deparaffinized sections in a glass slide were covered with Kodak Limited stripping film or apposed to emulsions. Organisms grown in a non-radioactive medium but otherwise identical, were processed simultaneously, placed on similar emulsions and exposed for equal time periods. Formvar coated emulsions were used to prevent direct contact of specimen and emulsion as a control to rule out pseudophotographic effects. Specimens were exposed for periods varying from 1 to 4 weeks, in black, light-tight, plastic boxes and kept in a refrigerator at about 5°C. Development was in D-19 solution for 4 minutes at 20°C. An acid stop bath of 1% acetic acid was used, and fixation was accomplished with 28% sodium thiosulfate. The specimens were washed, dried, and viewed unstained.

Results. Paramecia grown in the medium containing tritium gave well defined radioautographic images. The whole organism lying on the emulsion was sufficiently opaque to hide the underlying radioautographic image. If the organisms were removed, it was seen that a sharply defined radioautographic image was present. Emulsions apposed to organisms on a glass slide also gave sharp images.

Sections of paramecia cut at 1 μ gave the best autographs (Fig. 1). The sections were thin enough to permit the passage of light through the organism and to reveal the underlying emulsion image. Radioactivity was evident throughout the organisms both in endoplasm and ectoplasm. Many discrete round areas of concentration of tritium appear in the endoplasm. Most correspond to food vacuoles but a few very large areas resembled the macronucleus. The mouth was sharply outlined in some organisms. An exposure of one week gave good images. All controls were negative.

Yeast grown in the medium containing tritium and exposed for one week gave positive radioautographs on the most sensitive emulsions. Clusters of yeast showed an image in the radioautograph that corresponded identically to their pattern of arrangement on the slide apposed to emulsion. Usually a complete silhouette of each organism was given in the radioautograph when organisms were distinct (Fig. 2). Individual budding yeast cells were defined. Control yeasts did not cause reduction of emulsion when placed directly on emulsions, when placed on a Formvar coat overlying emulsions, or when apposed to emulsion.

Measurement of the smallest yeast cells by a filar micrometer eye piece using a calibrated stage micrometer showed sharp radioautographic images on the stripping film that were 3 μ in diameter. Sections of paramecium cut at 1 μ showed well resolved endoplasmic concentrations of radioactivity as small as 3 μ in the stripping film. Even smaller areas of radioactivity scattered between the paramecia were discernible but it could not be determined whether they were radioactive organisms or debris, or photographic artefact.

Discussion. Tritium, a radioactive isotope of hydrogen, emits beta particles having a maximum energy of 0.0179 Mev. and has a half life of 12.1 ± 0.5 years(7,8). Ninety per cent of its beta particles would be absorbed by one grain of silver bromide in nuclear track emulsions. It approaches the ideal for intercellular or intracellular localization. It is available at relatively little cost from the Atomic Energy Commission.

Its biological use as a label will depend on the particular compound under study. Tritium atoms bonded stably to carbon atoms can be used to label the latter just as the inactive hydrogen isotope deuterium has been used(9). In some compounds it may be easier to use tritium than to use radiocarbon.

Summary. Tritium, H^3, the radioactive isotope of hydrogen has been used for radioautography and given radioautographic images after a week's exposure of individual yeast

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cells and paramecia grown in media including sodium acetate containing tritium. Measured radioautographic images of endoplasmic concentrations of radioactivity in paramecium and of individual yeast cells of 3 μ have been delineated. Since the average beta particle from tritium is absorbed by a micron or less of nuclear track emulsion, from the standpoint of its range, this isotope approaches the ideal for radioautographic localization.

Yeast grown in tritium-containing medium as described and exposed for a shorter time have shown differential localization of tritium in polar and peripheral extranuclear areas. Some of these foci showed only one or two grains of reduced silver (each about 0.3 μ to 0.4 μ) in the emulsion. This probably represents the limit of resolution of radioautography with the present emulsions available for biological experiments.

We are indebted to the following members of the staff of Sloan-Kettering Institute: Dr. Leonard Hamilton for the paramecium, Dr. H. Christine Reilly for the yeast, and Mr. Mark Gettner for the thin sections.

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Apparent Discrepancies in Evaluation of Adrenocorticotrophic Hormone (ACTH) Activity by Two Assay Methods. (18732)

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The availability of a rapid and sensitive method for the assay of ACTH preparations by the adrenal ascorbic acid depletion test in the hypophysectomized rat(1) has made it possible to carry out comparative assays to an extent not allowed by the more time consuming procedure of assay by the adrenal maintenance test in the hypophysectomized rat(2). The lack of internationally accepted test procedures and preparations has, however, prevented the direct comparison of the level of activity of different samples of ACTH. Further than this, no detailed study has been made to date of: (a) the relationship between the potency of preparations in terms of ability to deplete ascorbic acid in the adrenal of the hypophysectomized rat, as compared with various morphological assay methods (adrenal repair and maintenance in the hypophysectomized rat), and (b) the relationship between the ascorbic acid depleting potency and the actual secretory activity of the adrenal gland in terms of the many and known experimental and clinical effects.

It is also known that the criteria (electrophoresis, solubility curves) for establishing the chemical homogeneity of hormone preparations are not strict enough to rule out trace contaminants (either anterior or posterior lobe) which may be pharmacologically active. Under such circumstances, it is obligatory that comparisons be made between various methods of assay, in order that the potency of various preparations can be evaluated in terms of types of responses, which may not necessarily be directly related to each other quantitatively. The present communication describes three series of experiments in which various types of ACTH preparations assayed by the ascorbic acid depletion test did not manifest a proportionally equivalent response in the adrenal maintenance test, or in secondarily manifested effects on the thymus and lymph nodes.

Methods and materials. Five preparations of ACTH were employed: A. ACTH protein prepared according to the method described in 1943(3), B. ACTH protein prepared according to a method to be described(4), C. ACTH peptide digestion mixture prepared from ACTH protein preparation (A), by a method described in 1948(5), (D) ACTH acid peptide in which the peptide is freed from the unhydrolyzed protein(6), and E. ACTH protein (B) treated with 0.2 N-HCl for 30 minutes in the boiling water bath.

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* Assisted by grants from the National Institutes of Health, U. S. Public Health Service, and from the Armour Laboratories and the Merck Co.

† Grateful acknowledgment is made to Mrs. Lorraine Anderson and to Mr. Harold Papkoff for assistance rendered.

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Assay of these preparations by the measurement of depletion of adrenal ascorbic acid was carried out on Sprague Dawley or Long-Evans rats,‡ hypophysectomized at 40 days of age, after 1 week in a constant temperature room, according to the technique of Sayers, *et al.*(1), and in similar animals injected for a period of 10 to 15 days beginning immediately after the day of operation, for the maintenance test(2). The latter groups of animals were maintained in a warm room under constant conditions and were fed this laboratory's wet Diet I, *ad lib.*, for the duration of the experiment. At autopsy, one adrenal gland, thyroid, thymus, testes and cervical lymph nodes were weighed on a torsion balance to the nearest milligram. Groups 1 and 2 were given identical ACTH preparations, the groups differing only in the strain of rats employed. Group 3 compares the maintenance responses of the standard (1943) protein at the 0.5 mg/day dose level with a protein preparation (1951) administered at a dose of 1 mg/day and having a higher ascorbic acid potency than the standard (1943) protein preparation. All preparations were tested for antidiuretic and pressor activity. The acid peptide D was substantially free of both activities whereas antidiuretic and pressor amounts in excess of 10 m.u./100 μg were present in the other preparations(7). None of the

TABLE I. Assay of Preparations of ACTH by Adrenal Maintenance and Ascorbic Acid Depletion Tests.

	Ascorbic acid depletion assay			Adrenal maintenance test in hypophysectomized rats							
							Body wt		Organ wt (and ranges)		
Treatment	Test dose, μg	Depletion of ascorbic acid mg/100 g ± SE	Stand. equivalent, μg	Duration of exp., days	Total dose, ACTH, mg	No. of rats	Initial, g	Gain or (loss), g	Adrenal (1) (right), mg	Thymus, mg	Cervical lymph nodes, mg
Group 1*—Sprague-Dawley rats											
Control (saline)	—	———	—	10	—	3	104	9.3	9.3 (7-10)§	268 (187-328)	91 (61-113)
ACTH protein (B)	5	118.5 ± 5.1 (14)†	5	10	5.25	5	110	10	16.2 (14-19)	125 (95-234)	69 (51-103)
,, Pepsin peptide (C)	2	112 ± 5.8 (22)	3.9	10	5.25	5	102	(1.2)	13.2 (11-15)	62 (32-142)	48 (44-61)
,, acid peptide (D)	2	148 ± 11.1 (9)	13	10	5.25	4	107	9.5	11.2 (11-13)	207 (138-267)	122 (101-149)
Group 2*—Long-Evans rats											
Control (saline)	—	———	—	11	—	7	132	(16)	4.4 (3-7)	210 (171-243)	150 (104-249)
ACTH protein (B)	5	118.5 ± 5.1 (14)	5	11	5.5	6	133	(19)	14.8 (12-17)	81 (27-139)	100 (80-130)
,, Pepsin peptide (C)	2	112 ± 5.8 (22)	3.9	11	5.5	6	131	(20)	13.6 (12-16)	69 (30-91)	108 (81-156)
,, acid peptide (D)	2	148 ± 11.1 (9)	13	11	5.5	6	135	(14)	10.8 (10-12)	144 (82-189)	126 (76-170)
Group 3*—Long-Evans rats											
Control (uninj)	—	———	—	15	—	6	117	(20)	3.7 (3-4)	143 (34-229)	119 (82-152)
ACTH protein (A)	5	119 ± 5.3 (11)	5	15	6.5	6	122	(11.5)	12.2 (11-14)	96 (58-112)	91 (65-132)
,, ,, (E)	5	141 ± 12 (7)	10.5	15	13.0	5	120	(15)	12.2 (9-15)	105 (41-225)	78 (68-87)

* All rats hypophysectomized at 40 days of age; intraperitoneal injections of 0.25 mg of each preparation were given twice daily (Groups 1 and 2) or once daily (0.5 or 1.0 mg daily) for 13 days (Group 3). Controls injected with buffered saline in equivalent amounts. Animals were examined under binocular microscope at time of autopsy for completeness of hypophysectomy.

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preparations exhibited thyrotropic or gonadotropic effects. The preparations were not tested specifically for growth, lactogenic or oxytocic activity.

Results. Table I presents the data for assay of ACTH preparations A-E by the ascorbic acid depletion and the adrenal maintenance tests in hypophysectomized rats. Test doses of ACTH preparations (B-E) are evaluated in terms of equivalent ascorbic acid depleting activity in micrograms of the standard protein A. The average weight of the right adrenal is presented in the table for each group of rats. These values can be compared by extrapolation with the standard curve employed in this laboratory(2) for dose-adrenal weight response and will be seen in every instance to show definite maintenance activity.

Examination of Table I will elicit the information that in groups 1 and 2, the ACTH protein (B) preparations were most active in the maintenance of adrenal weights, the acid hydrolysate (D) least active (although significantly greater than the controls), this in spite of a significantly greater ascorbic acid activity in the latter preparation. The most active preparation in terms of thymolytic and lympholytic activity, but with intermediate adrenal weight effect was the ACTH pepsin digest mixture (C) in groups 1 and 2. The ascorbic acid potency of this preparation is about twice that of the standard (1943) protein, although significantly less active ($p < .01$) than the acid peptide preparation.

In Group 3, 2 preparations are compared: the standard protein (A) at a dose level of 0.5 mg/day, as compared with a different protein preparation (E) of higher ascorbic acid activity and at a dose of 1.0 mg/day. The 2 treated groups show approximately the same effects on adrenal weight, thymus and lymph nodes, in spite of the difference in dose level and ascorbic acid depletion potency.

It may be noted that the preparation (D) exhibiting the highest ascorbic acid depleting potency is least active in causing reduction in weights of thymus and cervical lymph nodes. The suggestion must be made that high ascorbic acid depleting potency is not necessarily directly related to the secretion by the adrenal of substances causing reduction in thymus and lymph node weight.

The standard ACTH preparation described in 1943 was purified according to its assay either in the repair or maintenance of the hypophysectomized rat adrenal whereas recent ACTH preparations have been prepared using the ascorbic acid depletion test as a measure of purification.

The present data are interpreted to indicate the lack of a direct correlation between these assay methods.

The evidence suggests the necessity for parallel assays of ACTH preparations (proteins or peptides) by at least 2 methods until such a time as a correlation can be more clearly defined between (a) clinical and experimental observations and (b) activity as determined by various biochemical and morphological assay methods. The possibility must be noted that isolation by various chemical procedures of components of ACTH protein may result in the purification of a peptide exhibiting activity only in one or the other of the various test methods. Questions must also be raised as to (a) possible presence of two or more active factors, (b) differences in rate of absorption, utilization, and excretion, and, (c) role of trace contaminants.

Summary. Evidence is presented to indicate that the assessment of the potency of various types of ACTH preparations may vary according to the assay method employed. The suggestion is advanced that ACTH preparations be routinely assayed by more than one method (ascorbic acid depletion, adrenal repair and maintenance, effect on thymus, lymph nodes, etc.) in order to avoid misinterpretation of the nature of the activity and biological responses to a particular preparation.

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Some Factors Influencing Thymus Nitrogen Content of the Immature Male Mouse. (18733)

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White and Dougherty(1) have demonstrated that the adrenal cortex is involved in regulating the nitrogen content of lymphoid tissue. It is to be noted, however, that their results were obtained on pooled lymphoid tissue which included the thymus, spleen, and lymph nodes. No studies have been reported on the changes in nitrogen content of each individual type of lymphoid organ. Apparently, it has been assumed that all lymphoid tissues respond in an identical manner although the data of Andreasen(2) suggest that differences between the various lymphoid tissues may be expected.

We wish to report certain observations on the mouse thymus gland which reveal that neither the stress resulting from a 48 or 60-hour fast nor the short term administration of cortisone acetate has a significant effect on the total nitrogen content of the thymus, despite the fact that both the fasting and the cortisone administration produce a marked decrease in the weight of this organ. On the other hand, data are presented which indicate that certain other steroids can cause a significant decrease in the total nitrogen content of the thymus.

Immature male mice (Manor Farms), weighing between 13-17 g at the beginning of the experimental period, were used. Animals had access to water during the fasting period. Cortisone acetate was employed as an aqueous suspension; the other steroids were administered in oil solutions. The dosage and injection schedules are given in Table I. At the end of the experimental period, the mice were anesthetized with ether and the thymus and spleen of each animal were removed, weighed, and each organ used for Kjeldahl digestion. Semi-micro Kjeldahl analysis for total nitrogen was employed. Table I contains the data obtained from typical experiments. The fact that no significant decrease in thymus nitrogen occurs following fasting or cortisone administration, despite the marked weight loss, and raises the question as to what components are responsible for this loss. Preliminary observations indicate that it cannot be accounted for by a change in glycogen or "lipid" content.

White and Dougherty(1) observed a decrease in pooled lymphoid tissue nitrogen which occurred after fasting or after administration of adrenal cortical extract and this may be explained by the fact that the various lymphoid organs do not respond in the same manner or to the same degree. This is clearly evident from the data on the effect of fasting and cortisone treatment on spleen weight and total nitrogen content (Table I). A significant decrease in the total nitrogen content, as well as in the weight, occurs following both experimental situations.

It is particularly interesting that the steroids which reduce thymus nitrogen, in addition to their well-known endocrine effects, are also known protein anabolic compounds (3). These steroids do not appear to affect the nitrogen content of the spleen in any predictable manner as compared to their effect on thymus nitrogen. It is possible that a change in dosage or duration of administration will resolve this matter since the difference observed in the response of the two organs may be one of degree rather than kind.

The decreasing order of effectiveness of the various steroids, at the one dose level studied, in reducing the thymus nitrogen content (methylandrostanediol > testosterone propionate > β-estradiol > estrone > desoxycorticosterone acetate > methylandrostenediol > progesterone) is remarkably similar to the decreasing order of renotrophic (anabolic) effectiveness as reported by Kochakian(3) and confirmed in this laboratory (unpub-

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TABLE I. Influence of Fasting and Certain Steroids on Wt and Total Nitrogen Content of Thymus and Spleen.*

Treatment†	No. mice	Daily dose, mg	Total thymus N	Thymus wt	Total spleen N	Spleen wt
Control, fed	10	—	9.4 ± .7	250 ± 23	13.8 ± .7	410 ± 18
Fasted, 48 hr	10	—	10.0 ± .5	*153 ± 18*‡	*10.9 ± .9*	*265 ± 18*
Control, fed	10	—	10.6 ± .3	322 ± 20	13.8 ± .5	438 ± 32
Fasted, 60 hr	10	—	10.4 ± .7	*111 ± 19*	*11.4 ± 1.0*	*253 ± 32*
Control, saline	9	0.1 ml	7.3 ± .3	312 ± 10	11.7 ± .4	352 ± 14
Cortisone acetate	10	3.0	7.1 ± .7	*125 ± 7*	*7.3 ± .5*	*230 ± 21*
17-methylandrostane-3β, 17β-diol	8	1.5	*3.6 ± .2*	*158 ± 5*	*16.9 ± 1.2*	439 ± 31
Testosterone propionate	7	1.5	*5.2 ± .5*	216 ± 15	11.3 ± 1.2	365 ± 29
β-estradiol	8	1.5	*5.2 ± .3*	*159 ± 17*	13.4 ± 1.2	362 ± 36
Estrone	7	1.5	*6.1 ± .3*	*151 ± 18*	11.0 ± 1.0	293 ± 20
Desoxycorticosterone acetate	7	1.5	*6.4 ± .3*	*192 ± 13*	*8.6 ± 1.0*	302 ± 24
17-methyl-Δ^5-androstene-3β, 17β-diol	7	1.5	7.6 ± .3	207 ± 23	13.6 ± 2.3	445 ± 74
Progesterone	7	1.5	7.8 ± .4	219 ± 22	9.4 ± .7	299 ± 19
Testosterone succinate	6	1.5	7.9 ± .5	221 ± 16	12.2 ± 1.0	383 ± 19
Control, oil	7	0.1 ml	8.0 ± .6	255 ± 15	11.9 ± .8	337 ± 21

* Thymus and spleen wt expressed in mg/100 g body wt; thymus and spleen N as mg N/total organ/100 g body wt.

† All steroids inj. subcut. daily for 3 days.

‡ Italicized values are significantly different from their controls at the 5% level of confidence. Values reported are mean ± standard error.

lished). It appears quite possible that these results may form the basis for a suitable screening procedure for protein anabolic compounds. The physiological significance of this apparent relationship between thymus nitrogen depletion and protein anabolic steroid activity is being currently investigated.

Summary. Neither the stress resulting from a 48 or 60-hour fast nor the administration of cortisone acetate has a significant effect on the total nitrogen content of the mouse thymus gland. On the other hand, it appears that the protein anabolic steroids induce a significant decrease in the thymus total nitrogen content.

Addendum. After this work was completed, the paper by Adams and White(4) came to our attention. Their data, in agreement with our results, show that a 48-hour fast has no effect on the protein content of the thymus nodes.

The authors gratefully thank Miss L. Hopkins for assistance with some of the nitrogen determinations, Mr. S. Neuwirth for the statistical computations, and Dr. E. B. Hershberg for the cortisone acetate.

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A Purified Fraction of Thioflavine-S (Vasoflavine): Valuable Agent for Visualization of Blood Vessels.* (18734)

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A method for the visualization of blood vessels and lymphatics under ultraviolet light, using a fluorescent dye, Thioflavine-S, was developed by one of the authors (J.U.S.)(1). This technic has proved to be a valuable adjunct to the study of vascular physiology and morphology in the mesentery, intestines, uterus, and the kidney. The original method utilized the freezing-drying technic of Gersch (2) before cutting sections for microscopic visualization. Since that time the method has been adapted by Algire and Schlegel(3) for *in vivo* visualization through a transparent chamber, and has been simplified by Schlegel and Moses(4), eliminating the necessity for freezing and drying by clearing the tissue in glycerine.

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Thioflavine-S has been used for some time to stain the elementary bodies of the vaccinia virus(5), the potato scab(6), and as a fluorochrome stain of fixed tissue sections (7,8). Pick(9) tested several fluorescent dyes as to their efficacy as vital stains, but did not describe their ability to produce a fluorescence of the vascular walls.

He reports some experimental work in which Thioflavine in a concentration of 1:1000 was injected into the lymph sac and intracardially in the frog. It is doubtful if the blood vessels of the kidneys in his preparations would have shown any fluorescence because of the high dilution of the dye and the long period of time which elapsed before the organs were removed and inspected. (His shortest reported time between injection and observation was 5 minutes).

The technic employed in this study has been described at length in a previous publication(4), but will be briefly outlined herein. After anesthetizing the animal (in our case rabbits have been used for the most part) the abdomen is opened transversely. Hemostasis is secured and the wound is then held closed with clamps for approximately one-half hour to allow the animal to recover from the immediate effects of surgery. After this lapse of time the fluorescent dye, in a 4% solution, is injected intravenously in the amount of 1 cc per kg of body weight, and the kidneys removed within a period of 10 seconds after the onset of the injection. Sections of the fresh tissue are cut with a sharp knife and placed in glycerine (USP). The glycerine serves to clear the tissue and slow down post-mortem diffusion of the dye. It is important to use USP glycerine since excessive water promotes undesirable diffusion. The application of this method to the vessels of the rabbit kidney brings to light several interesting points. It was found that the rate of diffusion of the dye out of the kidney vessels is faster than from the vessels of the mesentery, subcutaneous tissue, and the uterus. It was also found that there was a very appreciable amount of natural fluorescence in the kidney, presumably from riboflavin and its derivatives. According to quantitative measurements by Mickelsen *et al.* (10) and Bessey *et al.*(11), the liver and kidneys of animals are uniformly higher in riboflavin content than other organs and tissues. This fluorescence was described by Ellinger (12) in the frog and the rat kidney. Attempts by him and by ourselves to eliminate this fluorescence by starvation and riboflavin deficient diets proved to be rather ineffective. This natural fluorescence appears to be greater in the dog kidney than in the rabbit kidney. In the rabbit kidney the natural fluorescence of the vessel walls is bluish-violet and of the parenchymal tissue yellow-green. The intravenous injection of Thioflavine-S makes the blood vessels fluorescent when observed in

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† Buswell Fellow in Urological Research.

‡ Graduate Research Fellow in Biochemistry supported by the Biological Stain Commission.

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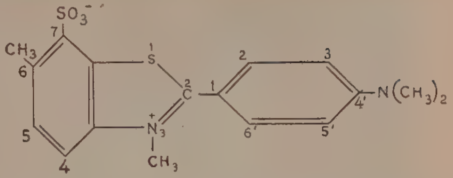
ultraviolet light, but the above factors tend to reduce the contrast between the vessels and the surrounding tissue. Since, as we have seen, there is little that can be done to influence the natural fluorescence, the problem of improving the contrast of the preparations was, of necessity, approached from the standpoint of the dye.

The commercial grade of Thioflavine-S shows both a yellow-green and blue-violet fluorescence. As seen in the kidney preparations from injected animals, the vessels and glomeruli fluoresce in the yellow-green and the surrounding tissues fluoresce in the blue-violet from the dye which has diffused out of the vessels. This is the exact opposite of the natural fluorescence. Keller(13) attributed this difference in fluorescence of Thioflavine-S to differences in "electric charge" of the tissues. Thioflavine-S, according to the Colour Index(14), is presumed to be composed of methylated and sulfonated derivatives of dehydrothio-p-toluidine. Synthesis is carried out with the primulin base as the starting material, successively methylated and sulfonated. This method can, understandably, produce a series of homologues, varying in degree of methylation and sulfonation, rather than any single pure dye. There is a strong possibility, however, that the major components are members of a limited series of the lower molecular weight homologues. Since it seemed probable that this mixture contained one or several compounds which would diffuse particularly slowly through the vessel walls, and since the yellow-green fluorescence was typical for the dye retained in the walls of the vessels, we were especially interested in separating this or these compounds from those with a higher rate of diffusion.

We took our problem to the research laboratory of the Eastman Kodak Co., where it was received with great interest and cooperation. It seems that the principal components of Thioflavine-S are the lower homologues of quaternarized, methylated, and sulfonated dehydrothio-p-toluidine, derived from the following basic structure.



The following compounds which are believed to be closely related to the components of commercial Thioflavine-S were prepared for our use:

A. Thioflavine-S, E-853-01 disulfonate. Anhydro-2-(3′-sulfo-4′-dimethyl aminophenyl)-3,6-dimethyl-7-sulfobenzothiazolium hydroxide.

B. Thioflavine-S, E-839-95A-monosulfonate. Anhydro-2-(4′-dimethyl aminophenyl)-3,6-dimethyl-7-sulfobenzothiazolium hydroxide.

C. Thioflavine-T, E-839-90A. 2-(4′-dimethyl aminophenyl)-3,6-dimethyl benzothiazolium chloride.

D. 2-(4′-aminophenyl)-6-methyl-7-sulfobenzothiazole, ammonium salt.

E. 2-(4′-amino-3′-sulfophenyl)-6-methyl-7-sulfobenzothiazole, disodium salt.

F. Primuline, recrystallized.

None of these substances when tested by our injection technic were as satisfactory from the standpoint of slowness of diffusion, or fluorescence, as the technical grade Thioflavine-S.

A more highly purified Thioflavine-S (Erie-Flavine-S conc. 130%)§ was obtained. A water extract of this sample was made by extracting 10 g of the dye with 50 cc of distilled water at room temperature. A 16% solution was obtained by this method and upon evaporation yielded approximately 8 g of dye. This water extract is supposedly composed of the more highly sulfonated fractions of Erieflavine-S. We have named the preparation Vasoflavine.¶ When injected intravenously into rabbits in the same concentration and over the same period of time, Vasoflavine proved to be more fluorescent and to diffuse less rapidly than Thioflavine-S. The contrast

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13. Keller, R., *Anat. Rec.*, 1949, v103, 133.

14. Colour Index, Ist Edition, Jan. 1924, Society of Dyers and Colourists, Bradford, Yorkshire, England.

§ Nat. Aniline Div., Allied Chemical and Dye Corp.

¶ May be obtained from the Pharmaceutical Laboratories of the National Aniline Division, Allied Chemical and Dye Corp., New York City.

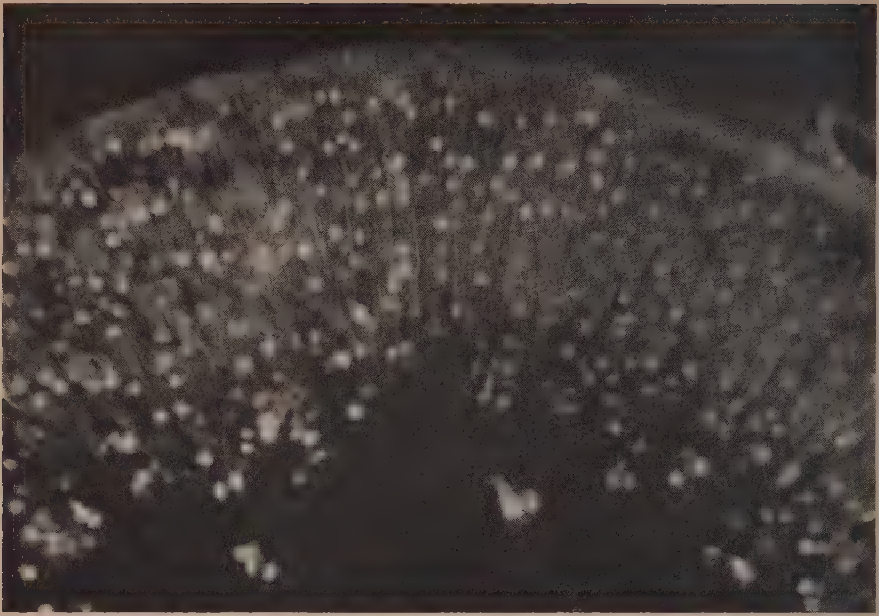

FIG. 1. Photomicrograph taken in incident ultraviolet light of a kidney from a rabbit injected intravenously with Thioflavine S. Note lack of contrast due to diffusion of the dye. 16.5×.

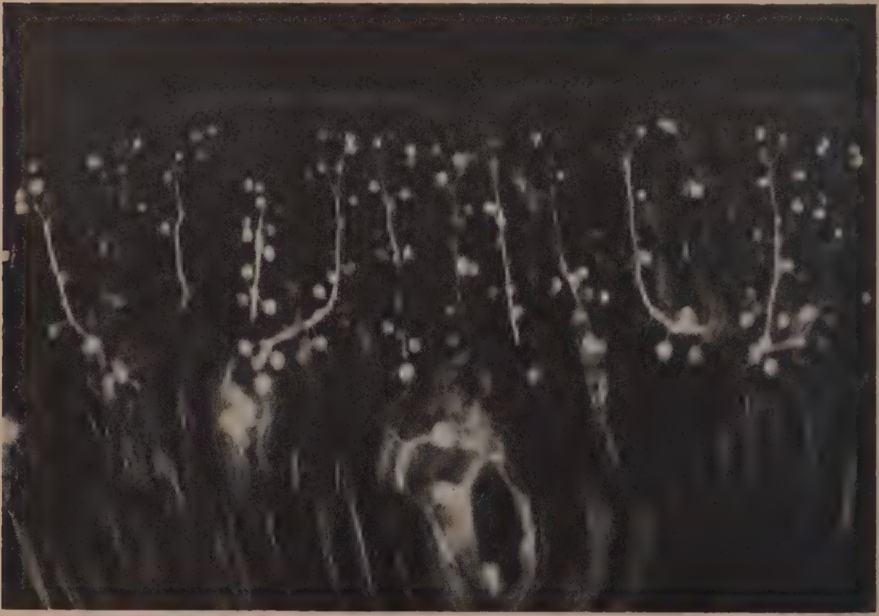

FIG. 2. Photomicrograph taken in incident ultraviolet light of a kidney from a rabbit injected intravenously with Vasoflavine. Note the excellent contrast, lack of diffusion of the dye, and the good injection of the smaller arterioles and vasa rectae. 16.5×.

provided with this preparation was thus very much improved. Small vessels, which with Thioflavine-S had been obscured by the natural fluorescence and by the diffused dye, were clearly visible with Vasoflavine. A comparison between the two dyes is shown in Fig. 1 and 2.

Since Vasoflavine more nearly approached the ideal dye for our purposes, it was of interest to determine the nature and character of this substance. Using the purified water extract as the experimental material, several studies were conducted to attempt more complete characterization of this dye.

Paper partition chromatographic studies. Paper partition chromatography was applied and many solvent systems were tested. These consisted of the more common ones such as: n-butanol-water; n-butanol with 10% acetic acid-water; n-butanol with 20% NH_4OH-water; phenol-water; ether-water; benzene-water; and collidine-water. None of these solvent systems produced good separation of any fractions, but most of them showed similar results which consisted of a slightly bluish fluorescent zone from the region of spotting on the chromatograph to the solvent front, while at the original spot or in the nearby region there was a highly fluorescent yellowish substance. It is doubtful that this was a result of the dye being fixed to the cellulose of the paper (this dye is known to be a direct cotton dye) for, by washing with water after development and drying of the chromatograph, the yellow fluorescent region was capable of further movement, and could be, at least partially eluted from the paper. The paper partition chromatograph did not give conclusive evidence as to whether more than one dye fraction was present, but rather it indirectly seemed to indicate that the dye may be found to exist in different forms which may account for the difference in intensity and color of the fluorescence under ultraviolet light.

It is a well known fact that practically all dyes readily form dimers and polymers especially in more concentrated solutions. Michaelis(15), Corrin(16), Rabinowitsch (17), and Sheppard(18,19) and others have conducted extensive studies which substantiate this. It seemed possible that the dimer or the higher polymeric forms are responsible for the yellow, highly fluorescent region near the original spot, while the monomeric form, which according to previous work should be the only form present in an alcohol solution, is the cause of the blue, slightly fluorescent region that moves with the n-butanol. Dialysis of the purified water extract of Erieflavine-S and spectra of the dye at various dilutions were studied to yield further evidence for the existence of polymeric forms.

Dialysis and spectrophotometric studies. Solutions of approximately 1% were continuously dialyzed for a week against distilled water which was changed 3 to 4 times daily. Viscose tubing (Visking Corp.) was used for dialysis. This retains compounds of molecular weight greater than 10,000-15,000. It was found that only a blue, slightly fluorescent substance was dialyzed, while the solution remaining within the tubing fluoresced yellowish-green under ultraviolet light. It is difficult to conceive why any of the dye should be retained unless it is partially composed of highly polymerized components. The dialysate and non-dialyzable material (hereafter called the non-dialysate) were evaporated to dryness on a steam bath. Fifty mg of each product was dissolved in 200 ml of distilled water. These two solutions were subjected to spectrophotometric analysis.‖ At the outset it was observed that the dialysate preparation had a considerably greater absorption in the region studied than the non-dialysate. Hence in the curves presented in Fig. 3, that for the dialysate represents a concentration one-quarter that for the non-dialysate. The spectral curves for the two fractions (Fig. 3) were fairly similar indicating that they were the

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15. Michaelis, L., *Symp. Quart. Biol.*, 1947, v12, 131.

16. Corrin, M. L. and Harkins, W. D., *J. Am. Chem. Soc.*, 1947, v69, 679.

17. Rabinowitsch, E. and Epstein, L. F., *J. Am. Chem. Soc.*, 1941, v63, 69.

18. Sheppard, S. E., *Rev. Mod. Physics*, 1942, v14, 303.

19. Sheppard, S. E., *J. Am. Chem. Soc.*, 1944, v66, 1995.

‖ Beckman, Model DU Spectrophotometer.

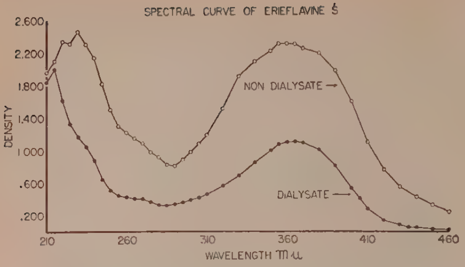

FIG. 3. Spectral curves of the dialysis products of Erieflavine-S purified, (Vasoflavine). Dialysate concentration 6.25 mg %, non-dialysate concentration 25 mg %.

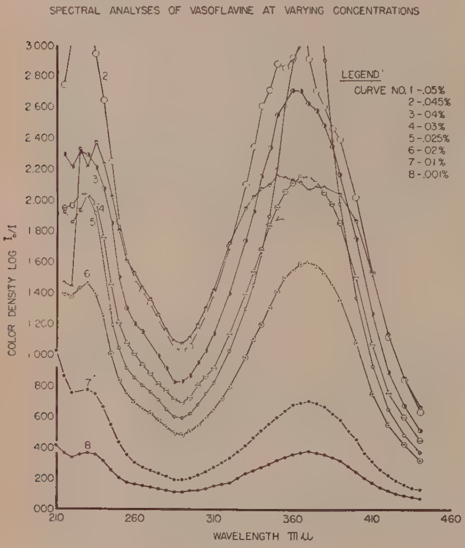

FIG. 4.

same dye (which was apparently quite homogeneous as concerns homologues), but there were slight differences in the 2 curves. There was a double peak for the non-dialysate (220 and 230 mμ) in the lower ultraviolet as compared with dialysate (215 mμ), and the major peak (360-365 mμ) was also flattened somewhat. The slight hump (330-340 mμ) on the side of the peak may be indicative of a dimer as illustrated by Michaelis(15) for other dyes. The curves do indicate that Vasoflavine is fairly homogeneous and does not consist of a series of homologues, since the curves show only two distinct principal absorption peaks which is common for many pure dyes. However, the difference in absorption coefficients of the dialysate and non-dialysate, and the variations of the spectral curves indicated that spectrophotometric analyses at varying concentrations of Vasoflavine were needed.

Spectrophotometric studies. A thorough study of spectrophotometric curves at various dye concentrations was conducted to determine whether the purified water extract, Vasoflavine, formed polymers in solution. A series of dilutions in distilled water ranging from 0.001% to 0.05% of Vasoflavine was made and spectral analyses were made on each from 210 to 440 mμ, using quartz cuvettes having a light path of 2 mm.

The results of these analyses are shown in Fig. 4. They indicate clearly that polymerization does occur to varying degrees, depending on the concentration of the dye. Upon analysis of the results it is seen that the curves are similar in shape at the lower concentrations (0.001-0.025%), having their major peak at 370 mμ and their minor peak at 230 mμ (Fig. 4, curves 5, 6, 7, and 8). According to Beer's law the density at these peaks increases proportionally as the concentration increases. This, however, is found to hold for this substance only in the lowest concentrations up to approximately 0.01%. The similarity of these curves is indicative that they represent spectral curves of the monomeric form of the dye. At higher concentrations (0.03%) it is noted that the density at 370 mμ decreases in spite of an increase in concentration (Fig. 4, curve 4). There is also a possible slight shift of the peaks toward the lower wave lengths. According to Michaelis and others(15-19), this is typical of the formation of a dimer or a higher polymeric form. When the concentration of the dye is increased to 0.04% (Fig. 4, curve 3) it is seen that the major and minor peaks shift further to lower wave lengths and the density is then increased with the increase in concentration. This is indicative of an increase in concentration of a higher polymeric form. At a still higher concentration of 0.045% (Fig. 4, curve 2) the amount of this form appears to be increased, but the appearance of another small peak at 350 mμ seems to indicate the beginning of a still higher polymer. When the concentration is increased even more to 0.05% the curve shows two peaks (Fig. 4, curve 1), which are greatly flattened and shifted from those of the monomeric and other lower forms in both the major and minor peak regions. The density is reduced from that of the 0.045% solution, indicating the formation of one or more new polymeric forms with lower absorption coefficients.

The data obtained from this study clearly indicate that Vasoflavine is capable of forming polymers, dependent upon the dye concentration. The dye apparently exists in polymeric forms at concentrations greater than 0.025%, and even higher polymers appear to exist above 0.04%.

Upon calculation of the possible concentration of Vasoflavine in the blood of injected rabbits according to our regular technic, it was found that the best results, with less diffusion out of the vessels, were obtained when the dye concentration in the blood was greater than 0.03%, assuming that the dye dispersed rapidly throughout the blood stream. It seems therefore reasonable to believe that the dimeric or polymeric form of the dye is the more desirable, for it diffuses from the blood vessels less readily and apparently imparts a more brilliant yellow-green fluorescence to the blood vessel walls.

Summary and conclusions. A more fluorescent and less rapidly diffusible dye with a strong affinity for the walls of the blood vessels has been described. This dye is a purified, water soluble fraction of Thioflavine-S and has been named Vasoflavine. Studies leading to a more accurate characterization of this dye are described. These include paper chromatography, dialysis, and spectrophotometry.

The following conclusions are offered: 1. Vasoflavine approaches the ideal dye for the visualization of blood vessels under ultraviolet light and is much superior to the Thioflavine-S previously used. 2. The solution of dye probably exists as an equilibrium between monomeric and various polymeric forms. 3. The higher the concentration of an aqueous solution of the dye the more of the polymeric forms exist. 4. The yellow-green fluorescence is apparently produced by the higher form and the bluish by the monomeric form. 5. The polymers are probably too large in molecular size to pass the barrier of the vessel walls readily, while the monomeric form quickly diffuses out of the vessels, thus explaining the yellow-green fluorescence of vessels and the bluish fluorescence of the surrounding tissue. It should be noted that Vasoflavine exhibits no apparent toxic effects in experimental animals (rats, rabbits, and dogs) in blood concentrations as high as 275 mg %, but extensive toxicity studies have not been carried out.

The authors wish to acknowledge the very kind cooperation and assistance of Dr. Burt H. Carroll, Dr. William West, Dr. C. F. H. Allen, Mr. Vernon Saunders, and Mr. Robert Sprague of the Research Laboratories of Eastman Kodak Company in the preparation of derivatives of Thioflavine-S tested by us, and of the National Aniline Division of the Allied Chemical and Dye Corporation, who generously supplied us with the commercial and purified Erie-flavine-S. We are indebted to Dr. Elmer Stotz, Professor of Biochemistry, for his helpful suggestions in studies to aid in the characterization of the dye, and invaluable assistance in the writing of this paper. Credit is also due to Miss Anita R. Kushner for the excellence of her work with the photographic material.

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Mechanism of Action of Mercurial Diuretics. (18735)

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Gremels(1) observed that mercurial and xanthine diuretics increase the oxygen consumption of the isolated dog kidney perfused with blood from a heart-lung preparation.

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1. Gremels, H., *Arch. Exp. Path. u. Pharmakol.*, 1929, v140, 205.

Farah and Maresh(2) have shown that the diuretic action of mersalyl is inhibited by the administration of half a mole of dimercaptopropanol (BAL) for every mole of mersalyl. This finding suggested to these authors "that the possible mechanism of action of the mercurial diuretics is the inactivation of one or more SH-containing enzyme systems responsible directly or indirectly for reabsorption of salt and water from the lumen of the kidney tubules."

Among the SH-containing enzymes the succinic oxidase system has been studied extensively and methods for its determination are available(3). It appeared desirable to study the respiration of tissue slices as well as the activity of the succinic oxidase and other SH-containing enzyme systems in the kidney of the rat at the height of mercurial diuresis. Although our studies are still in progress we feel it is desirable to submit this preliminary report, particularly in view of the recent publication of Handley and Lavik(4), in which they state that "it may well be that the succinic dehydrogenase system of the kidney is involved in the active reabsorption of water and sodium chloride by the cells of the renal tubule."

Procedure. Mersalyl* was used as the mercurial diuretic for the experiments described below.

a. *Pre-diuretic control experiment.* Albino rats weighing 150-250 g were used. Food and water were withheld from all animals for 15 hours prior to the time they were used for an experiment (5 p.m. to 8 a.m.). The left kidney was removed under ether anesthesia and determinations were made of the respiration of cortical tissue by the conventional Warburg technic, and of the succinic oxidase activity by the method of Schneider and Potter(3). A week later, when the abdominal wound was healed, the right kidney was removed under the same conditions and the same determinations were made. It was found that the respiration as well as the succinic oxidase activity of the right kidney remained the same and thus each animal could be used as its own control.

b. *Diuretic experiment.* The respiration and succinic oxidase activity of the left kidney was determined as above. A week later 10 mg per kg body weight of mersalyl (*i.e.* 4 mg Hg/kg) dissolved in 2 cc saline were injected into the tail vein. Simultaneously 50 cc saline per kg body weight were injected intraperitoneally and the kidney was removed three hours later. The control rats were injected only with the same volume of saline, and the right kidney was also removed after 3 hours. The details of this method of assay will be published separately. At present it can be said that this method of administration of the mercurial results in profuse diuresis in almost every single rat as compared to the controls receiving only saline. The diuresis reaches its maximum in 2 to 3 hours. Most of the fluid is excreted within 8 hours although the effect of the diuretic is still apparent after 10 hours. Lipschitz *et al.*(5) devised a method of assay giving the mersalyl intraperitoneally and they observed a diuretic effect only after 5 to 10 hours even with the simultaneous administration of ammonium chloride. Dicker (6) administered mersalyl intramuscularly to rats and observed the diuretic effect 10 hours after the injection.

Results. The results of our experiments are summarized in Table I. It will be seen that the standard diuretic dose of 10 mg mersalyl (4 mg Hg/kg) had no significant effect either on the respiration of cortical tissue slices or on the succinic oxidase activity. Handley and Lavik, using a technic different from ours reported a 40% decrease in the succinic oxidase activity after injecting 8 mg of Hg or double the dose we used. We have tried to repeat Handley's experiments(4) using mersalyl, but as can be seen from the last two columns of

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2. Farah, A., and Maresh, G., *J. Pharmacol.*, 1948, v92, 73.

3. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1943, v149, 217.

4. Handley, C. A., and Lavik, P. S., *J. Pharmacol.*, 1950, v100, 115.

* Mersalyl was generously supplied by the British Drug Houses Ltd. and the Sterling-Winthrop Research Institute.

5. Lipschitz, W. L., Hadidian, Z. and Kerpósar, A., *J. Pharmacol.*, 1943, v79, 97.

6. Dicker, S. E., *Brit. J. Pharmacol.*, 1946, vI, 194.

TABLE I. Effect of Mersalyl on Respiration of Rat Kidney Slices and on Succinic Oxidase Activity of Homogenates at Height of Diuresis. (For Explanation See Text.)

No. of animals	Mersalyl, mg/kg	Q_{O_2} O_2 L. kidney Mean	Stand. dev.*	Q_{O_2} O_2 R. kidney Mean	Stand. dev.	Q_{O_2} (succin.) L. kidney Mean	Stand. dev.	Q_{O_2} (succin.) R. kidney Mean	Stand. dev.
13	—	19.1	1.1	19.1	1.3				
19	10	19.8	1.04	18.8	1.1				
10	—					187	7.6	188	8.7
15	10					183	5.1	188	8.2
4	—					196	8	190	9.6
10	20					182	14.2	163	16.1

* $\sqrt{\frac{\Sigma d^2}{n-1}}$

Table I, the decrease in the succinic oxidase activity observed was statistically insignificant. The maximum inhibition obtained in one case was 22%. It has been observed that after injection of the mercurial the kidney swells and if no dry-weight determination is done on that particular kidney misleading results could be obtained.

In our experience, a dose of mersalyl representing 8 mg Hg per kg is not a diuretic dose for the unilaterally nephrectomized rat. Lipschitz *et al.* have called attention to the narrow dosage range for the diuretic activity of mercurials. The technic followed by Handley and Lavik would not permit them to ascertain whether the dose they used produces diuresis. When we gave the high dose of 8 mg Hg/kg to unilaterally nephrectomized rats under the conditions reported above, we found that at least half of the animals showed oliguria as compared to the controls. The kidneys were removed a day after the administration of the mercurial and sent for histopathological examination. It was found that with this high dose of mercury all rats showed extensive necrosis of the proximal convoluted tubules, involving about 60% of the cells. Even with the standard dose of 4 mg Hg/kg some animals showed definite irreversible damage which was much milder than with the greater dose. A dose of 5 mg mersalyl (2 mg Hg/kg) produced no pathological change in unilaterally nephrectomized rats and no diuresis was observed.

Discussion. The fact that diuresis caused by mercurials can be inhibited by dimercaptopropanol (BAL) is no proof that mercurial diuretics act by inhibiting SH-containing enzymes. It only means that BAL binds the mercury more firmly than do the tissues. Mercurials can react with proteins other than through SH-groupings, and even if certain SH-containing enzymes are inhibited by therapeutic doses of mercury it does not necessarily follow that SH-containing enzymes are involved in the reabsorption of salt and water from the renal tubules. The question as to whether or not a certain enzyme, be it SH-containing or not, is involved in the reabsorption of salt and water can best be answered by observing whether a known specific inhibitor of that enzyme can produce diuresis. It would then be necessary to reach a high concentration of that inhibitor in the kidney tissue. The heart-lung kidney preparation of the dog is convenient for this purpose and work is proceeding in this laboratory to test the effect of such inhibitors on diuresis.

Summary. 1. Mersalyl given intravenously in therapeutic doses has no influence on the respiration of kidney cortical slices of the rat at the height of diuresis. 2. Under the same conditions mersalyl has no effect on the succinic oxidase activity of cortical homogenates. 3. The question of participation of enzymes in the reabsorption of salt and water from the renal tubules is discussed.

We are indebted to Dr. Edmund Shwayri for the histopathological studies.

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Relationship of Pituitary and Adrenal Cortex to Ovarian Hyperemia Reaction in the Rat.[*] (18736)

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Ovarian hyperemia in response to the injection of pregnancy urine described by Reiprich(1) has been found sufficiently reproducible in rats and mice to warrant its use as a standard test for pregnancy(2) and for the assay of chorionic gonadotrophin and of certain pituitary extracts. The significance of this reaction in ovarian physiology, however, is little understood for, though it apparently is an early indication of ovarian stimulation in some species, the part that it plays in ovarian function is not known.

The inability of human chorionic gonadotrophin (HCG) to produce ovarian hyperemia in the hypophysectomized animal unless a large dose of pituitary "synergist" is given has been shown(3). Further studies on the influence of the pituitary and on the role of the adrenal cortex in this phenomenon are presented in this study.

TABLE I. Effect of Various Substances on Production of Ovarian Hyperemia in Intact and Hypophysectomized Rats.

Intact	No. of animals	
	Positive	Negative
Controls (no inj.)	2	40
Cortisone acetate, 1.25 mg	0	2
HCG, 0.1 μg + cortisone acetate, 1.25 mg	0	2
HCG, .02 mg	36	8
Pituitary extr., 3.0 mg	12	0
Corticotropin, 10 mg	0	2
Hypophysectomized		
HCG, .02 mg	2	13
HCG, .5 mg	0	2
Pituitary extract, 3.0 mg	4	0
Cortisone acetate, 1.25 mg	0	3
HCG, .02 mg + cortisone acetate, 1.25 mg	15	1
HCG, .02 mg + corticotropin, 1 mg	7	1
HCG, .02 mg + corticotropin, .5 mg	2	2

Methods. Weanling 35-45 g female rats of the Sprague-Dawley strain were used. Hypophysectomy or adrenalectomy were performed 16 to 20 hours before the injection of the test substances, which were given subcutaneously over the abdomen. When more than one injection was given, they were placed on opposite sides of the abdomen. Four hours after injection the animals were killed with chloroform and ovarian color was judged independently by two or more observers under a daylight fluorescent lamp. The ovaries were classified as positive or negative as they would be judged in the interpretation of a pregnancy test. Doubtful reactions were classified as negative. A positive response on one side combined with a doubtful response on the other was regarded as a positive test. The combination of a weakly positive and a negative reaction was considered a negative test. Intact uninjected and gonadotrophin-treated animals were included as controls in each day's experiment. The corticotropin used(4) has been found to be 25 times as active as the Armour standard La-1-A and contained no significant amounts of gonadotrophins or prolactin.

Results. The minimal effective dose for ovarian hyperemia of the preparation of human chorionic gonadotrophin used was 1 μg,[†] and to insure maximal responses a dose of 20 μg was employed except where otherwise stated. The minimal effective dose of the anterior pituitary preparation used[‡] was 0.1 mg

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[*] We are indebted to Dr. Karl Folkers of Merck and Co., Rahway, N. J., for most of the steroids used in this study.

1. Reiprich, W., *Klin. Wchnschr.*, 1933, v12, 1441.

2. Salmon, U. J., Geist, S. H., Salmon, A. A., and Frank, I. L., *J. Clin. Endocrinol.*, 1942, v2, 167.

3. Fried, P. H., Rakoff, A. E., *J. Clin. Endocrinol.*, 1950, v10, 423.

4. Payne, R. W., Raben, M. S., and Astwood, E. B., *J. Biol. Chem.*, 1950, v187, 719.

[†] "Pranturon" generously supplied by Schering Corporation, Bloomfield, N. J.

[‡] Antuitrin T, No. 099802, kindly supplied by Dr. D. A. McGinty of Parke, Davis and Co., Detroit, Mich.

TABLE II. Effect of Steroid Hormones and Gonadotrophins on Ovarian Hyperemia in Adrenalectomized Rats.

	No. of animals	
Adrenalectomized	Positive	Negative
HCG, .02 mg	8	54
Cortisone acetate, 1.25 mg	0	2
Pituitary extract, 3.0 mg	0	9
” ” 3.0 ” + cortisone acetate, 1.25 mg	17	1
HCG, .02 mg + cortisone acetate, 1.25 mg	25	3
” ” + cortisone acetate, 100 μg	5	1
” ” + cortisone acetate, 10 μg	8	2
” ” + cortisone (free alcohol), 10 μg	17	4
” ” + cortisone (free alcohol), 3 μg	0	7
” ” + compound F acetate, 100 μg	5	3
” ” + compound F acetate, 10 μg	4	0
” ” + compound F acetate, 3 μg	0	4
” ” + compound A acetate, 100 μg	7	4
” ” + compound A acetate, 10 μg	8	6
” ” + isocortisone acetate, 10 μg	4	8
” ” + Lipoadrenal Cortex, .1 cc	4	1

and a dose of 3 mg was used throughout. As shown in Table I, cortisone acetate alone in doses of 1.25 mg did not produce ovarian hyperemia in intact, adrenalectomized, or hypophysectomized animals. Corticotropin in doses as high as 10 mg did not produce ovarian hyperemia in intact animals.

No synergism between cortisone acetate and human chorionic gonadotrophin was seen in intact animals when sub-hyperemic doses of human chorionic gonadotrophin (0.1 μg) were given with large doses of cortisone acetate.

Removal of the pituitary blocked ovarian hyperemia in response to chorionic gonadotrophin in doses as high as 0.5 mg but did not abolish hyperemia brought about by the anterior pituitary preparation. Cortisone acetate and corticotropin restored the response to chorionic gonadotrophin in hypophysectomized animals.

Table II shows that adrenalectomy abolished the hyperemia response both to human chorionic gonadotrophin and to the anterior pituitary extract. Cortisone acetate restored this response to both substances. Doses of cortisone acetate, free cortisone, and compound F acetate as small as 10 μg were found to be effective. Iso-cortisone acetate and compound A acetate were effective in slightly larger doses. Lipoadrenal Cortex (Upjohn) was effective in a dose of 0.1 cc, the smallest dose tested.

Progesterone in oil in a dose of 1 mg produced ovarian hyperemia in adrenalectomized chorionic gonadotrophic treated animals as did desoxycorticosterone at a dose of 2.5 mg (Table III). Testosterone propionate in oil, methyl androstenediol in oil, stilbestrol in oil, estradiol in oil, and pregnenolone as an aqueous suspension were not effective in this regard in the doses tested.

The data presented indicate that secretions of the adrenal cortex are necessary for the production of ovarian hyperemia in the rat in response to both human chorionic gonadotrophin and pituitary extract. Also, the effect of corticotropin in restoring the ovarian hyperemia response appears to be mediated through the adrenal cortex.

Summary. Adrenalectomy and hypophy-

TABLE III. Effect of Other Steroids on the Production of Ovarian Hyperemia in Adrenalectomized Immature Rats.

	No. of animals	
Adrenalectomized	Positive	Negative
HCG, .02 mg + progesterone in oil, 2.5 mg	2	0
” ” + progesterone in oil, 1.0 mg	5	2
” ” + progesterone in oil, .1 mg	0	5
” ” + progesterone in oil, .01 mg	1	5
” ” + desoxycorticosterone acetate in oil, 2.5 mg	2	0
” ” + desoxycorticosterone acetate in oil, 1 mg	0	5
” ” + testosterone propionate in oil, 2.5 mg	1	4
” ” + testosterone propionate, in oil, 1.0 mg	1	3
” ” + methyl androstenediol in oil, 1 mg	1	4
” ” + stilbestrol in oil, 2.5 mg	0	3
” ” + estradiol in oil, 1 mg	1	4
” ” + pregnenolone, 2.5 mg	0	2

sectomy were found to abolish the ovarian hyperemia response to human chorionic gonadotrophin. Corticotropin and cortisone acetate restored this effect in hypophysectomized rats, and cortisone acetate, as well as other steroids, in the adrenalectomized rats. Adrenalectomy but not hypophysectomy prevented this response to an anterior pituitary preparation. Cortisone acetate reinstituted the effect in the adrenalectomized anterior pituitary injected animals.

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Heme Synthesis and Erythrocyte Life Span in the Cat.* (18737)

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The interpretation of the hematologic syndromes resulting from exposure to ionizing radiations depends in large measure on the life span of the formed elements in the circulating blood(1,2). The rate of utilization of leukocytes(3) and thrombocytes(4) has already been determined for the cat. In other experiments(5,6) the functional regeneration of myeloid and erythroid elements in the cat was compared. In order to interpret this work a measure of the life span of the red cell in this species was needed.

While a variety of methods have been used for determining erythrocyte life span, the method of isotope tagging of the porphyrin ring is probably the least objectionable and was the one chosen.

Material and methods. Five apparently healthy adult cats from the stock colony, immune to infectious feline agranulocytosis, were used. They were maintained on a diet of canned commercial dog food, a small weekly allotment of fresh horse meat and an admixture of ground Rockland Rat Chow. Each was given 100 mg/kg of glycine-N^{15} containing 63.3 atoms % excess of N^{15}. The isotopic glycine divided into 15 doses was fed over a period of 3 days. The animals weighed 2.7 to 3.1 kg with a mean of 2.9 kg. Erythrocyte counts, white blood cell counts and hemoglobin values were determined weekly. Blood samples for analysis were obtained at about 2 week intervals by placing the animals under light ether anesthesia and cutting down on the femoral artery with the usual aseptic precautions. The blood samples were drawn in oxalate, the packed red cells re-suspended in physiological saline, re-centrifuged and finally lysed with distilled water. Hemin was obtained from the lysed cells by routine procedures(7,8). The hemin N^{15} concentrations were determined on a Consolidated-Nier Isotope Ratio Mass Spectrometer. The blood volume of each cat was determined at the end of the experiment by the method of Evans Blue dilution.

Results. During the course of the work it became apparent that individual cats gave somewhat different hemin N^{15} concentrations at a time when their red cell heme was maxi-

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* This paper is based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

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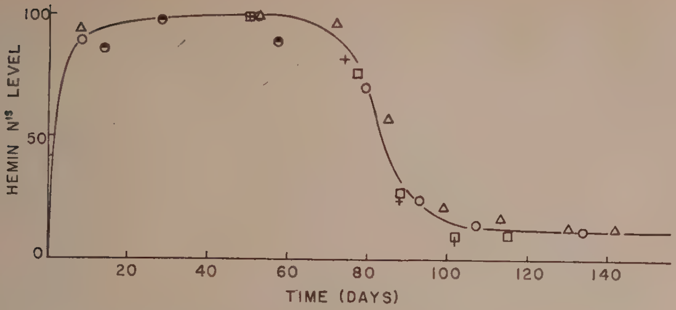

FIG. 1. Composite curve for 5 animals derived from corrected hemin N^{15} excess ratios expressed as per cent of plateau values.

mally labeled (about 50 days). Further, it was apparent that repeated sampling of blood, the volume of each sample being approximately 10% of the circulating blood volume, was in itself decreasing the hemin isotope level. Accordingly, two corrections were applied to the data to make them more comparable and to provide a mean curve from which the half-life of the red cell could be calculated. First each value was multiplied by a factor derived from the sample volume and the cat's blood volume, itself corrected for dilution by previous sampling. Second, observed N^{15} excess ratios, corrected for sampling, were expressed as percentages of the plateau N^{15} level for that animal. The corrected data are presented in Fig. 1.

Using the method of calculation presented by Shemin and Rittenberg(9), the mean life span, corrected for the initial labeling period, was determined to be 77 days, while half of the cells had a life span between 72½ and 81½ days.

Summary. The mean life span of the erythrocyte in the cat as determined by labeling heme with N^{15} is 77 days.

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Passive Sensitization of Human Skin by Sera of Guinea Pigs Anaphylactically Sensitized to Ovalbumin. (18738)

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Previous studies have shown that immediate urticarial reactions of the Prausnitz-Kustner type may be produced in human skin after passive sensitization with sera from rabbits sensitized with ovalbumin and with sera from guinea pigs sensitized to pollens of grass or ragweed(1,2). The present study concerns the activity in passively sensitizing human skin of sera from guinea pigs sensitized with crystalline ovalbumin, and the relationship of that activity to the capacity of the same antisera to produce passive anaphylactic sensitivity in the guinea pig.

Samples of sera obtained from guinea pigs sensitized by various methods at the Allergen Research Division of the Department of Agriculture were tested in the passive sensitization of human skin at Roosevelt Hospital. Other

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portions of the same antisera were tested in the passive anaphylaxis of guinea pigs at the Allergen Research Division.

Methods. To determine activity in passive anaphylaxis, serial dilutions of each serum (differing by a factor of 1.5) were injected intraperitoneally into normal guinea pigs weighing 280-310 g. These animals were challenged 2 days later with intravenous injections of 1 mg of ovalbumin. Assays were considered satisfactory if 2 of the serial dilutions produced nonfatal reactions and the 2 succeeding lower dilutions produced fatal anaphylaxis. The anaphylactic titer was expressed in terms of the number of fatal sensitizing doses per ml of serum. Skin-sensitizing activity was determined by injecting 0.1 ml of serum intracutaneously on the back or upper arm of a normal human test subject, shown by previous skin tests not to be allergic to ovalbumin or guinea pig serum. Twenty-four to 48 hours later 0.05 mg of ovalbumin, dissolved in 0.025 ml of saline, was injected intracutaneously into the same site. All sera were tested on two different volunteers. Reactions were observed after 15 minutes. Sites showing positive reactions were retested in serial dilutions to measure the skin-sensitizing antibody, the titer being the highest dilution producing a distinct reaction.

Sera from 8 guinea pigs sensitized to ovalbumin, which showed anaphylactic titers of 13-40 fatal sensitizing doses per ml, all failed to passively sensitize human skin. These animals were sensitized by single subcutaneous injection of 0.002-0.3 mg of alum-precipitated ovalbumin. They were bled 3-32 weeks after the sensitizing injection. Sera from 2 guinea pigs sensitized with 3 such subcutaneous injections (0.3 mg each) and bled 4 weeks after the last dose likewise failed to sensitize human skin, but showed anaphylactic titers of 13 and 20. Two guinea pigs were sensitized by intraperitoneal injections of ovalbumin solution in saline. One was bled 4 weeks and the other 32 weeks later. Anaphylactic titer of the first serum was less than 1.7; the titer of the second serum was 5. These antisera showed no skin-sensitizing activity.

Another series of 9 ovalbumin antisera was prepared from 7 guinea pigs that had survived anaphylactic shock, either once or twice. (Table I). These animals had been sensitized 6 to 8 weeks previously with a single subcutaneous injection of 0.02-0.3 mg of alum-precipitated ovalbumin. All 7 guinea pigs were bled 6 to 13 days after surviving a moderate or severe shock induced by intravenous injection of 0.077-0.59 mg of ovalbumin. Two of these guinea pigs survived a second intravenous challenging injection of 1 mg of ovalbumin 18 days after the first and were again bled 11 days after the second shock.

TABLE I. Skin-sensitizing and Anaphylactic Activity of Sera of Guinea Pigs Surviving Challenging Doses of Ovalbumin.

Guinea pig No.	Sensitizing dose,* mg	Interval before challenge	Challenging dose,† mg	Interval before bleeding, days	Anaphylactic titre	Skin sensitizing titre
160	.02	6 wk	.077	6	40	0
195	.02	6 ʼʼ	.260	6	100	1-100
180	.3	6 ʼʼ	.173	11	133	0
181	.3	6 ʼʼ	.115	11	100	0
706	.3	8 ʼʼ	.39	13	130	1-10
	s. c.‡	18 days	1.0	11	200	1-10
707	.3	8 ʼʼ	.59	13	400	0
711	.3	8 ʼʼ	.26	13	200	1-100
	s. c.	18 days	1.0	11	130	1-10

* Alum precipitated ovalbumin subcut.

† Intrav., in saline sol.

‡ s. c. = second challenge.

All 9 antisera induced passive anaphylactic shock; the titers ranged from 40 to 400 doses per ml. Antisera from 3 of the 7 guinea pigs passively sensitized human skin in dilutions of 1:10 to 1:100, the other 4 did not, even when undiluted. There was no significant difference either in anaphylactic titer or skin-sensitizing capacity between the sera prepared after the first, and after the second challenging injection. No relationship between anaphylactic titer and skin-sensitizing capacity was apparent.

Thus, sera from some guinea pigs sensitized with ovalbumin produced passive sensitization of human skin, but sera from others similarly treated were inactive in this respect. All of the antisera showing skin-sensitizing activity were from guinea pigs which had survived anaphylactic shock following challenging injections. However, no antisera were obtained from these pigs before the shock dose. Hence, the effect of the challenging injection on the formation of this type of antibody could not be assessed.

The skin-sensitizing activity of the sera bore no relation to their potency in passive sensitization of guinea pigs. This observation permits the speculation that skin-sensitization and anaphylaxis are mediated by different antibodies, the skin-sensitizing antibody being formed by only a fraction of the guinea pigs in response to a type of stimulation which consistently produced anaphylactic antibody.

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Certain Relationships Between Pteroylglutamic Acid, Citrovorum Factor, and Cortisone. (18739)

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Recent investigations have shown that relatively large amounts of dehydroisoandrosterone were interchangeable with pteroylglutamic acid (PGA) for growth of *Streptococcus faecalis* and *Lactobacillus casei*(1). In view of these observations cortisone was tested with *S. faecalis* for its ability to replace PGA and with *Leuconostoc citrovorum* for its ability to replace citrovorum factor (CF)(2), a substance related chemically to PGA(3). Cortisone was found to replace both PGA and CF for the two organisms mentioned; moreover, it reversed the inhibitory effects of Aminopterin, a potent antagonist for both PGA and CF.*

Experimental. The cultures used were *Le. citrovorum* ATCC 8081 and *S. faecalis* ATCC 8043. The medium used for *Le. citrovorum* was that of Sauberlich and Baumann (2) substituting acid-hydrolyzed casein for part of the amino acid mixture. The basal medium of Rabinowitz and Snell(5) was used for *S. faecalis*. The organisms were grown at 37°C, growth being observed turbidimetrically at 20 hours using a Coleman Model 11A spectrophotometer. Both the clinical aqueous suspensions and crystalline preparations of cortisone were used.† Leucovorin, a synthetic substance with CF activity(3), was used as a source of CF.

Results and discussion. Preliminary experiments indicated that the method of dissolving and diluting cortisone had a marked influence on the amounts required to give growth of the organisms. Table I shows the results obtained with a variety of sample treatments. The mild acid treatment was used to destroy any citrovorum factor possibly occurring in the samples of cortisone. No evidence for the presence of CF was found. The acid treatment consistently enhanced the potency of the clinical cortisone preparation while in 2 experiments the acid treatment decreased the potency of the crystalline cortisone sample.

The most reproducible results were obtained by evaporating alcoholic solutions of crystalline cortisone in the assay tubes. In several experiments by this technic 1.5 γ per ml gave

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* Preliminary experiments on replacement of PGA and CF by cortisone were discussed at Federation Meetings, 1951(4).

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† The clinical suspension was "Cortone Merck". A sample of crystalline cortisone acetate was obtained through the courtesy of Merck and Co.

TABLE I. Amounts of Various Cortisone Preparations Required to Give Growth of *Leuconostoc citrovorum* and *Streptococcus faecalis.*

Treatment	Amt needed in γ/ml of culture medium for ½ max growth* *Le. citrovorum*	*S. faecalis*
Clinical cortisone		
Suspended in water	25, 40	
” ” †	45	30
Dissolved in Tween 40 and steamed 30 min. at pH 2	10, 25, 50	
Crystalline cortisone		
Dissolved in Tween 40	1.5, 7.5, 15	50
Dissolved in Tween 40 and heated	1.5, 2.7, 7.5	300
Dissolved in Tween 40 and autoclaved 5 min. at pH 2	50, 75, 100	100
Dissolved in alcohol and evaporated in assay tube	1.5, 2.5, 4.0	5

* Optical density of 0.55 on Coleman Spectrophotometer.

† Microbiological test medium contained Tween 40.

TABLE II. Comparison of Amounts of Cortisone, Leucovorin, and Pteroylglutamic Acid Needed for Growth of *Le. citrovorum* and *S. faecalis.*

Compound	Amt needed in γ/ml of culture medium for one-half max growth *Le. citrovorum*	*S. faecalis*
Cortisone*	1.5	5.0
Leucovorin	0.00015	.0004
Pteroylglutamic acid	30.0	.0002

* Crystalline product dissolved in alcohol and evaporated to dryness.

half-maximum growth of *Le. citrovorum.* Larger amounts of all the preparations of crystalline cortisone were required by *S. faecalis* to replace PGA.

In an occasional experiment with *Le. citrovorum,* particularly in the earlier phases of this study, cortisone in any amount tested failed to replace CF. The reason for this has not been determined.

Table II compares the amounts of cortisone, PGA and leucovorin needed for growth of the organisms. Although 10,000 times more cortisone than leucovorin is required by *Le. citrovorum,* cortisone is 20 times more potent for this organism than PGA, which is closely related chemically to leucovorin. Dehydroisoandrosterone which replaces PGA for *S. faecalis*(1) was found to replace CF for *Le. citrovorum* at 50 γ per ml.

The mechanism whereby cortisone replaces PGA and CF was studied by use of Aminopterin (4-aminopteroylglutamic acid). Cortisone eliminated the growth inhibition of *Le. citrovorum* produced by toxic amounts of Aminopterin. The ratio of metabolite:antagonist for half-maximum growth was changed from 0.0037 to 0.0025 by a four-fold increase in cortisone concentration (Fig. 1). This comparatively small change indicated that the relationship between cortisone and Aminopterin was competitive and that cortisone is probably a catalyst rather than an end product of the enzyme reaction inhibited by Aminopterin.

Rodney *et al.*(6) have shown that livers from rats rendered PGA-deficient by inclusion of 2% succinylsulfathiazole in their diet had a lowered capacity to oxidize tyrosine. These workers later showed that the oxidation of tyrosine can be partially restored *in vitro* by PGA(7). Rienits(8) has found that PGA will stimulate the disappearance of tyrosine from liver preparations from scorbutic guinea pigs. To test the effect of cortisone on tyrosine oxidation, albino rats (Carworth Farms) were fed for 5 weeks a purified diet(9) containing 1% succinylsulfathiazole but no PGA.

TABLE III. Effect of Cortisone on Oxidation of Tyrosine by Rat Liver Homogenates.*

	Rat fed diet			
	Without PGA		With PGA	
Time, min.	Tyrosine	Tyrosine + cortisone	Tyrosine	Tyrosine + cortisone
30	3†	35	7	30
60	12	58	15	55
80	17	72	15	65

* Flasks contain 2 ml of 14% liver homogenate in .067 M phosphate buffer. .1 mg cortisone was added to flask in alcohol and alcohol evaporated off. .5 mg tyrosine was added to side arm and tipped in after 10 min equilibration.

† Difference in oxygen uptake (μl O_2) between that of liver homogenates and liver homogenates plus test substances.

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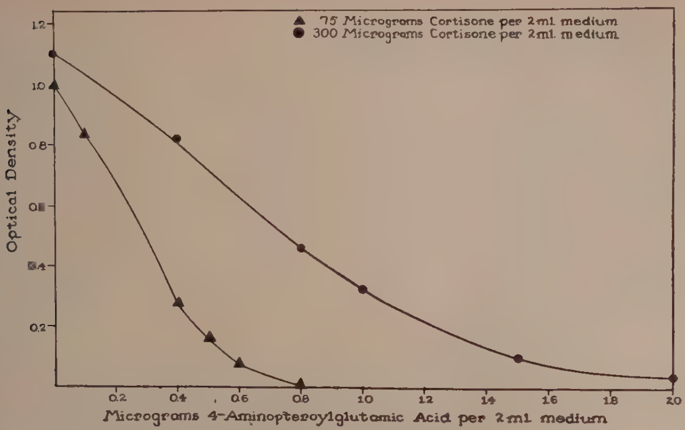

FIG. 1. Effect of Cortisone on inhibition of growth of *Leuconostoc citrovorum* by Aminopterin.

Control rats were fed the same diet with added PGA. As shown in Table III crystalline cortisone markedly stimulated the oxidation of tyrosine by liver homogenates from rats on both diets. Cortisone had no effect on endogenous respiration *i.e.* without added tyrosine. PGA and leucovorin had no discernible effect on tyrosine oxidation by liver homogenates from either group of rats. Presumably the rats fed the succinylsulfathiazole were not deficient enough to demonstrate the reported effect of PGA *in vitro*. The magnitude of the cortisone stimulation of tyrosine oxidation appears to be considerably greater than that reported for PGA(7).

Summary. 1. Cortisone was found to replace pteroylglutamic acid and citrovorum factor for growth of *Streptococcus faecalis* and *Leuconostoc citrovorum*, respectively. Cortisone reversed the growth inhibition of *Le. citrovorum* produced by toxic amounts of Aminopterin. 2. Cortisone *in vitro* stimulated the oxidation of tyrosine by liver homogenates from rats fed a purified diet containing 1% succinylsulfathiazole with or without PGA.

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Effect of Parenterally Administered Glycine upon Action of Insulin in Rabbits.* (18740)

R. A. YOUNGMAN AND RALPH HASKINS. (Introduced by D. M. Pace.)

From the Lincoln Clinics, Lincoln, Neb.

Varying reports have been made of the effect of amino acids given orally and parenterally upon the blood glucose level. Izumi(1) found that glycine administered intravenously caused a slight rise in the level of glucose in the blood and that glycine administered perorally caused a slight lowering over a four-hour period. Schenck(2) found that 1 g of glycine administered to an animal perorally produced a hypoglycemia, whereas 1.5 g by the same route produced a hypoglycemia. This effect was more intense in an animal weighing 2810 g than in one weighing 1260 g. Hypoglycemia was produced in man by administration of glycine by the same route according

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* This study was aided by a grant from the Nebraska Medical Foundation.

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to Schenck. Johlin(3) has reported the effect of parenteral administration of glycine on the sensitivity of rabbits to insulin. According to his findings, the injection of 3 mg of glycine per kg in rabbits produced an increased sensitivity to insulin. This could be demonstrated at 3-week and 10-week intervals by producing insulin shock with half the usual convulsive dose. Studying this problem from another aspect, Cunningham, Barnes, and Todd(4) reported that oral feeding of glycine to rats, not only maintained the liver glycogen content in greater concentration after administration of insulin, but also tended to prevent hypoglycemia.

In view of the conflicting reports of the effect of glycine administration on the blood glucose level, it was felt worth while to repeat the work of Johlin to see whether his findings might be verified. We have accordingly repeated Johlin's work, varying the procedure slightly.

Procedure. Nearly grown male albino rabbits were used as test animals. These animals were all fed the same standard diet. Each rabbit was fasted 18 hours before being tested. A fasting blood sugar was determined. Insulin was then administered in the dosage of 0.7 unit per kg. Blood sugar determinations were then made hourly, for 5 hours, following the administration of insulin. Following the final blood sugar determination, glycine was administered intravenously in the amount of 3 mg/kg. In 3 weeks the same procedure was repeated except that no further glycine was administered.

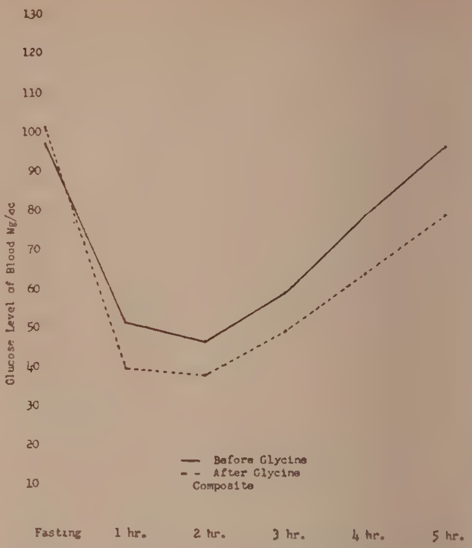

FIG. 1. Composite graph showing effect of equivalent amounts of insulin on blood glucose level before and after glycine.

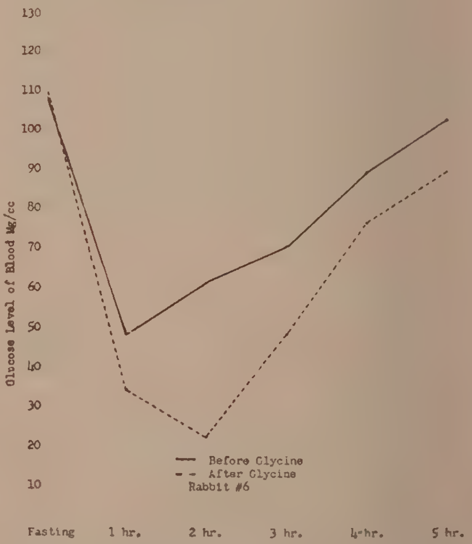

FIG. 2. Graph showing effect in a single rabbit of equivalent amounts of insulin on blood glucose level before and after glycine.

Our results substantially bear out those of Johlin, in that we found a fairly consistent reduction in the blood sugar levels following the administration of insulin. The increased hypoglycemia began within the first hour following insulin and persisted for 5 hours. The composite results from 7 rabbits have been prepared in graph form (Fig. 1). For these 7 animals the average fasting blood sugar preceding administration of glycine was 97 mg per 100 cc. One hour after subcutaneous administration of 0.7 unit of insulin per kg, the blood sugar fell to a mean of 50.7 mg. At the end of the second, third, fourth, and fifth hours, the blood sugar was 46.2 mg, 58.7 mg,

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78.1 mg, and 95.2, respectively. Three weeks after administration of glycine to these animals a mean fasting blood sugar of 101 mg was found. The same dose of insulin according to weight was then given and blood sugar determinations were made hourly. At the end of one hour the blood sugar was 39 mg. At the second, third, fourth, and fifth hours it was 37.4 mg, 48.5 mg, 63.5 mg, and 78.7 mg, respectively. This gives in rough figures a decrease of approximately 10 mg after the first hour and extending to the fifth hour. In the case of animal No. 6 (Fig. 2) the effect was somewhat more striking. Prior to the administration of glycine in this animal the fasting blood sugar was 110 mg. After administration of insulin the lowest blood sugar, 48 mg, as shown in the accompanying graph, occurred at the end of one hour. It rose gradually to 102 mg 5 hours after insulin. When retested 3 weeks after the administration of glycine, a fasting blood sugar of 113 mg was found. Following administration of insulin the lowest blood sugar level was 22 mg occurring 2 hours after insulin and rising to 89 mg 5 hours after insulin.

There was considerable variation in the response of different animals. Rabbit No. 9, for some reason not apparent had a consistently higher blood sugar following the administration of glycine than prior to it. If this animal is excluded, results are considerably more impressive.

Why a normally occurring constituent of the body should have this effect or why it should have any effect when given in these relatively small amounts is not apparent. There are instances in which the body controlling mechanism may be disrupted by the administration of excessive amounts of normally occurring substances.

Summary. 1. The effect of parenteral administration of glycine in rabbits on the effect of insulin is reported. 2. Parenteral glycine under certain conditions renders rabbits more sensitive to insulin.

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Early Histological Changes Following Obstruction of Pancreatic Ducts in Dogs: Correlation with Serum Amylase. (18741)

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Clinical diagnosis of pancreatitis depends largely upon detection of elevated serum amylase levels. The relationship between these and the extent or duration of early pancreatic changes remains relatively obscure. After ligation of the pancreatic ducts in experimental animals, previous investigators have noted a transient rise in serum amylase reaching a maximum during the subsequent few days(1). In cats fasting 48 hours, pancreatic ligation produced no histologic evidence of inflammatory reaction in the pancreas unless stimulants such as secretin or parasympathomimetic drugs were given(2). The early microscopic changes resulting from duct ligation plus stimulation were widening of the septa and presence of inflammatory cells in these spaces. More severe reactions showed inflammatory cells within the lobules and destruction of acinar cells. In rabbits fasted 48 hours before the experiment, vacuolation of some acinar cells and dilatation of a few ducts were present 24 hours after ligation. After 11

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* Most of the studies here reported were made during tenure of a National Research Council Senior Fellowship in Pediatrics, under a grant from the National Foundation for Infantile Paralysis.

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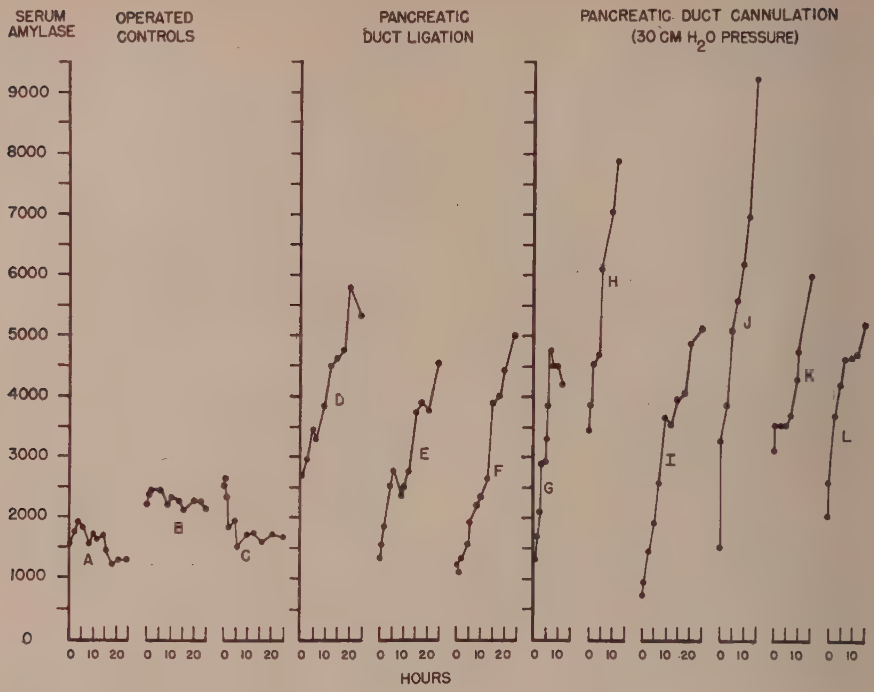

FIG. 1. Changes of serum amylase following pancreatic duct ligation, pancreatic duct cannulation with 30 cm H_2O pressure, and in operated control dogs.

days most of the acinar cells had degenerated and many ducts had dilated(3).

The present study was undertaken to provide a closer correlation between histological findings and serum amylase levels during the first 24 hours following ligation of the pancreatic ducts. Because the pressure within the ligated ducts was not known, the duct systems of other dogs were cannulated and subjected to a definite pressure of 30 cm H_2O by means of a reservoir of sterile saline. This pressure had been found to approximate the maximum which could be produced physiologically by means of repeated injections of secretin. On other dogs, serving as operated controls, the pancreatic ducts were exposed but not ligated or cannulated.

Experimental details. The dogs were last fed approximately 20 hours prior to the experiment. They were kept under anesthesia by occasional intravenous injections of sodium pentobarbital for the entire experimental period of 24 hours (or less). Serum amylase was determined by a modification of the Somogyi method with use of additional NaCl, a phosphate buffer to maintain a pH of 6.8(4), copper sulfate-sodium tungstate deproteinization and the alkaline copper sugar reagent of Somogyi(5). Results were expressed as mg glucose equivalent released per 100 cc serum in 30 minutes.

At the end of the experimental periods, tissue blocks were cut from the pancreas *in situ,* fixed in formalin and stained with hematoxylin and eosin.

Results. The changes in serum amylase activity are shown in Fig. 1. No significant alteration of serum amylase concentration occurred in the operated control dogs A, B, C. No edema of the pancreas was noted grossly. Histologically, there appeared to be no spreading of the interlobular septa. A few inflam-

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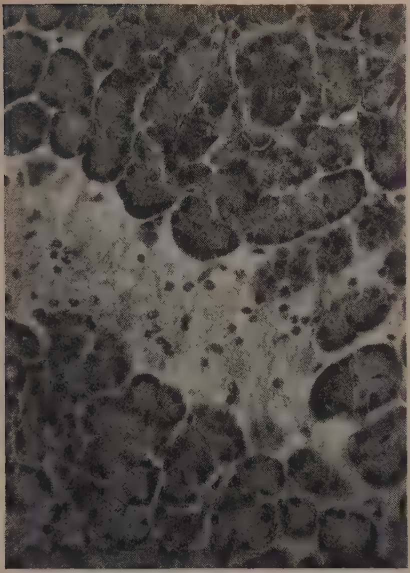

Fig. 2. Inflammatory cells in distended interlobular septum of pancreas, 24 hr following ligation of pancreatic ducts. (Dog D, Fig. 1). ×280.

matory cells were in the capsule.

In the dogs D, E, F with ligation of both pancreatic ducts, a gradual rise of serum amylase occurred throughout the 24-hour period of observation. By the end of that period a value 2.8 times the initial value was reached in the average of the 3 dogs. By 5 to 7 hours the pancreas appeared to be definitely edematous. At 24 hours the histological studies showed spreading of the interlobular septa and inflammatory cells in these spaces as illustrated in Fig. 2. Edema induced spreading also of the capsule. The leukocytic infiltration of the capsule was more marked than in the operated control dogs. No leukocytes appeared among the acini. No noteworthy degeneration of acinar cells, separation of acini or dilatation within acini appeared. The acinar cells were well stocked with zymogen granules. The slightness of the histological alteration suggests that absorption of enzyme-bearing fluid into the blood stream without actual structural damage may account for the elevation of serum amylase.

In dogs G-L, in which the pancreatic duct system was placed under pressure of 30 cm water, the rise in serum amylase was more rapid. Dogs J, K, L died at approximately 15 hours. In Dog I the rise of serum amylase was slower and the dog survived for the full 24 hours. Dogs G and H were killed at 12 hours. Shock was marked in all for many hours. The average rise of serum amylase in 12 hours in the 5 dogs was to a value 2.7 times the initial value. Thus the rise in serum amylase was approximately twice as rapid in the dogs with artificial pressure as it was in those where an indefinite amount of pressure developed following simple ligation of the ducts. In the dogs with artificial pressure, the pancreatic edema appeared earlier and more markedly than in the ligated dogs. Fat necrosis was extremely slight, and that mainly in areas traumatized by dissection or rubbing with respiratory movements. Some fluid exuded from the surface of the pancreas in most instances and accumulated in the peritoneal cavity. No definite point of leakage could be detected. Microscopically, there was more intense infiltration of inflammatory cells into the interlobular spaces. The most consistent and distinctive feature of the histological change in this group of dogs was the presence of inflammatory cells among the acini (Fig. 3). In some instances there were foci of infiltration with total disappearance of acinar cells. Some of these foci were in the vicinity of small ducts and in the 2 instances, L and K, there were leukocytes in the ducts. Vacuo-

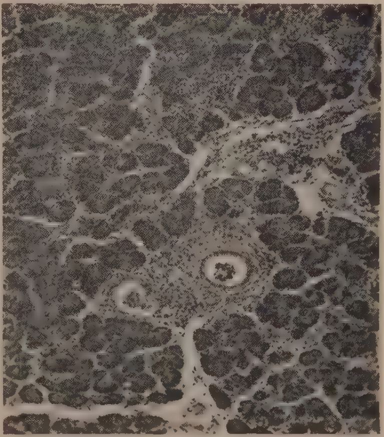

Fig. 3. Inflammatory cells in septa, among acini, and in pancreatic ducts after 15 hr of intraductal pressure of 30 cm H_2O. (Dog K, Fig. 1). ×140.

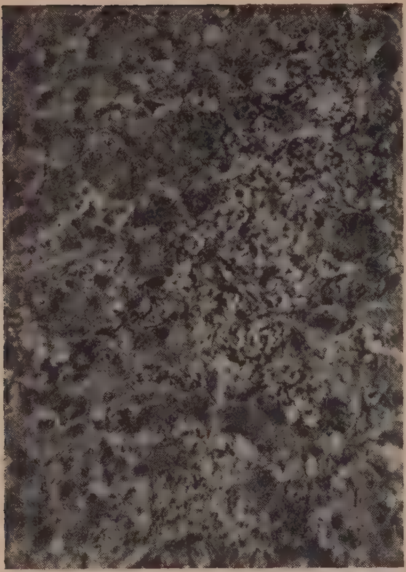

Fig. 4. Degeneration of acinar cells after 15 hr of intraductal pressure of 30 cm H_2O. (Dog J, Fig. 1). ×280.

lation of acinar cytoplasm was limited largely to the group with artificial pressure. In dog J, this progressed to an extreme degree. In small areas the staining properties of the acinar cells were completely abnormal but little or no inflammatory reaction was observed, in association with this change (Fig. 4). In most other areas, however, the cells appeared normal and well stocked with zymogen granules. In most of the dogs subjected to definite pressure, erythrocytes appeared in the interlobular spaces and in one instance infiltrated an acinar area. There was thus a pancreatitis which might be described as hemorrhagic as well as purulent or necrotic.

Summary. Both pancreatic ducts were ligated in 3 dogs. Over a period of 24 hours a gradual rise of serum amylase and a moderate edema with a slight leukocytic infiltration of the interlobular connective tissue of the pancreas occurred. The pancreatic duct systems of five dogs were subjected to a pressure of 30 cm water. Serum amylase rose twice as fast as in the dogs with simple ligation. Histologically, the principal changes in the pancreas were a more intense leukocytic infiltration, including acinar areas, and degeneration of some acinar cells.

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Electron Microscope Study of Anaplasmosis in Bovine Red Blood Cells. (18742)

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In a recent work on the virus host relationships of the foot and mouth disease virus with erythrocytes a technic was developed by which regular blood smears of the infected red cell membranes can be observed under the electron microscope (Epstein, Fonseca, and De Robertis(1). This technic was found to be useful for the study of parasites of erythrocytes. Some preliminary observations on piroplasmosis and especially on anaplasmosis will be reported here.

The *Anaplasma marginalis* Theiler, 1909 is well suited for electron microscope research since it is only about 0.4 to 1 μ in diameter. It is generally described as a round marginal body simply constituted of a single mass of chromatin (Fig. 1a), with no cytoplasm and multiplying by binary division(2). Recently there has been a tendency to consider the

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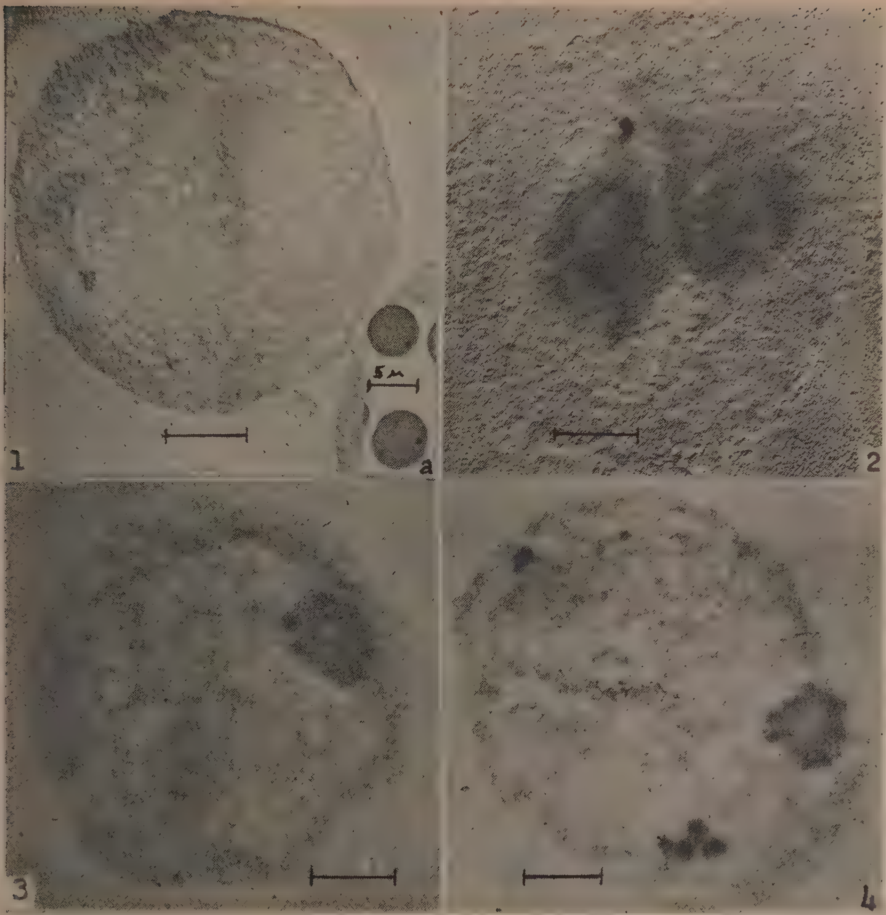

FIG. 1. Normal control red cell membrane from a calf. 11,500×. a. Erythrocytes infected with *Anaplasma marginalis*. Giemsa stain. Optical microscope. 1500×.

FIG. 2. Red cell membrane of a calf infected with *Piroplasma bigeminum*. 11,500×.

FIG. 3. Red cell membrane of a calf infected with *Anaplasma marginalis*. The mass of the parasite appears undivided in the center and divided into round bodies at the periphery. 11,500×.

FIG. 4. The same as Fig. 3. In addition to the marginal parasite 5 elementary bodies are dispersed within the erythrocyte. 11,500×.

anaplasma to be bacterial in nature because of its form, lack of cytoplasm, and the reaction with the Feulgen reagent(2,3). However, the most accepted view is to consider it within the group of other intracellular parasites of protozoan nature(2).

Diagnosis of this disease can be easily made in acute cases by microscopic examination of blood smears, since the marginal bodies are found in a relatively high concentration. However, in chronic cases the diagnosis is frequently questionable because the concentration of marginal bodies is very low and they may resemble other bodies found in normal red blood cells(4).

Experimental. Five calves were inoculated

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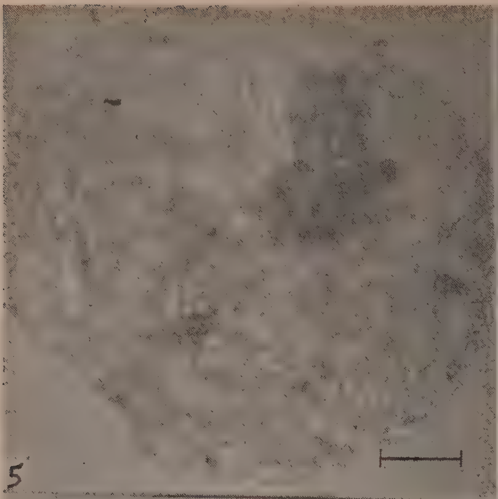
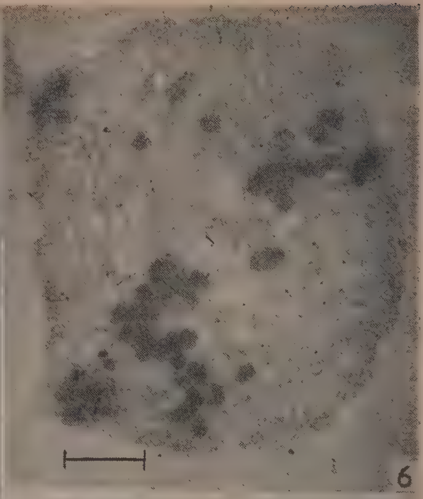
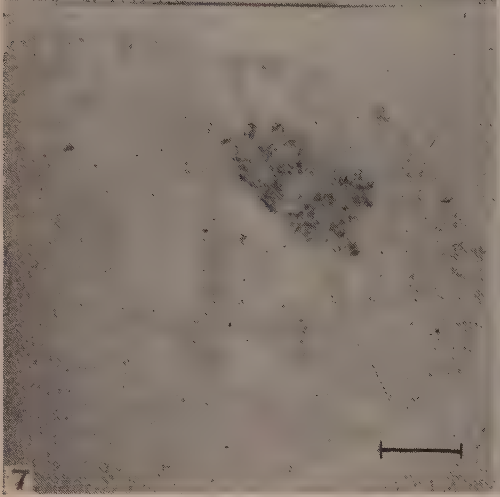
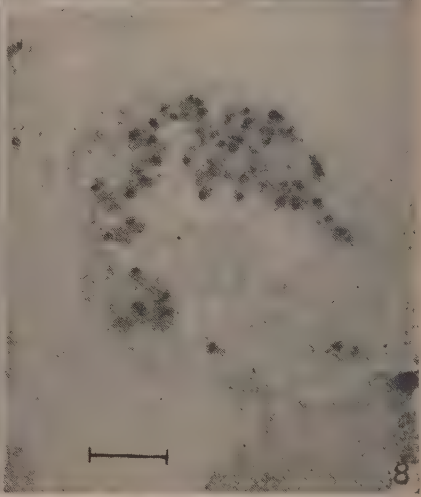

FIG. 5. The same as Fig. 4. The entire mass of the parasite is divided into elementary bodies. 11,500×.

FIG. 6. In addition to an undivided mass (lower left) a large number of bodies are dispersed throughout the erythrocyte. 11,500×.

FIG. 7. Red cell membrane of a calf infected with *Anaplasma marginalis* and treated with "Pirevan." The mass of the parasite has low electron density and the limits become indistinct. Granular material of very high electron density is deposited within the parasite. 11,000×.

FIG. 8. Similar to Fig. 7. Granular dense material deposited within the elementary bodies. 11,000×.

Fig. 1, 2, 5, 7, and 8 are shadowcast with palladium at an angle of 11°.

with virus obtained from the Instituto de Biología Animal of Uruguay, which consisted of citrated blood obtained during the acute stage of the disease. Ten ml of this blood were injected subcutaneously. Typical clinical and hematological symptoms were obtained after an incubation period of 28 days. Blood smears stained with current hematological stains were studied at different times after inoculation. In one case during the acute state the calf was treated with one injection of "Pirevan" (Evans) containing 5% of Dimethylquinolil-methyl-sulfate-urea. A few experiments with *Piroplasma bigeminum* were also carried out on calves. For the electron microscope study, blood smears were hemolyzed with distilled water and the red cell membranes were transferred on standard nickel grids by a parlodion replica technic. Grids were examined directly or after light shadowing with palladium at an angle of 11°. An R.C.A. EMU 2C electron microscope was used.

Results. A normal bovine red cell membrane is shown in Fig. 1. It appears as a flat disc of about 10 mμ in thickness, showing very fine structural details on the surface.

Fig. 2 shows a typical *Piroplasma bigeminum* with a pair of pear shaped masses. Some indications of internal structure are observed but the parasites are too large for detailed electron microscopy.

Typical *Anaplasma marginalis* are illustrated in Fig. 3 and 4. They consist of a round mass of about 1 μ which shows clear indication of an internal structure. The central part of the parasite seems rather undivided but at the periphery there are rounded bodies ranging in diameter from 170 to 220 mμ which protrude on the surface of the parasite. In addition to this composite structure of the *anaplasma* itself the electron microscope reveals bodies dispersed within the red blood cell which are essentially similar to those within the parasite. As a matter of fact observing the sequence of Fig. 4, 5, 6 and many more unpublished electron micrographs, the general impression is obtained that a large number of elementary bodies can be formed and become dispersed throughout the erythrocyte. Fig. 5 is interpreted as probably representing two attached marginal bodies and it seems justified to assume that at the level of the optical microscope they could be interpreted as a parasite dividing by binary fission.

On Fig. 6 in addition to about 40 elementary bodies dispersed throughout the red cell membrane an undivided mass of the parasite of about 0.8 μ can be observed.

The diameter of 119 elementary bodies was measured and the results expressed in a distribution curve (Fig. 9A). The measurements range between 170 to 430 mμ with about 69% of the total ranging between 170 and 220 mμ. These results were also distributed into 2 groups: (1) elementary bodies belonging to the mass of the *anaplasma* (Fig. 9B); (2) elementary bodies distributed throughout the erythrocyte (Fig. 9C). It can be seen that in this last group there is a wider range of distribution with some bodies which are definitely larger than those localized within the *anaplasma*.

Fig. 7 and 8 show some parasites which were found after a single injection of Pirevan together with those of normal appearance. With the available information it is difficult to advance any interpretation of these figures and to establish if they are related to the treatment. On purely morphological bases one can observe a granular material of very high electron density which seems to be precipitated strictly within the limits of the parasites. As seen in Fig. 7 and 8, in addition to this very dense material, the parasites themselves have lost their typical electron density and the boundaries of the elementary bodies fade out and become undistinct.

Discussion. The preliminary results presented here tend to indicate that it would be of considerable interest to make a systematic study with the electron microscope of intracellular parasites, and particularly of those attacking the red blood cells. The limiting factor is the large size of some parasites, such as the *Piroplasma bigeminum,* which makes it very difficult to reveal any internal submicroscopic structure.

The electron microscope study of *Anaplasma marginalis* reveals 2 interesting features. One is the observation that the parasites, which appear at the level of the optical microscope as single masses (Fig. 1a), are generally constituted of a central undivided mass of high electron density and by rounded peripheral bodies of 170 to 220 mμ in diameter. In some cases the whole mass of the parasite appears to be constituted of tightly packed elementary bodies. The second is the fact that in addition to the bodies present within the mass of the parasite, a great number of them are dispersed throughout the erythrocyte. Most of these elementary bodies are definitely submicroscopic (Fig. 9) and

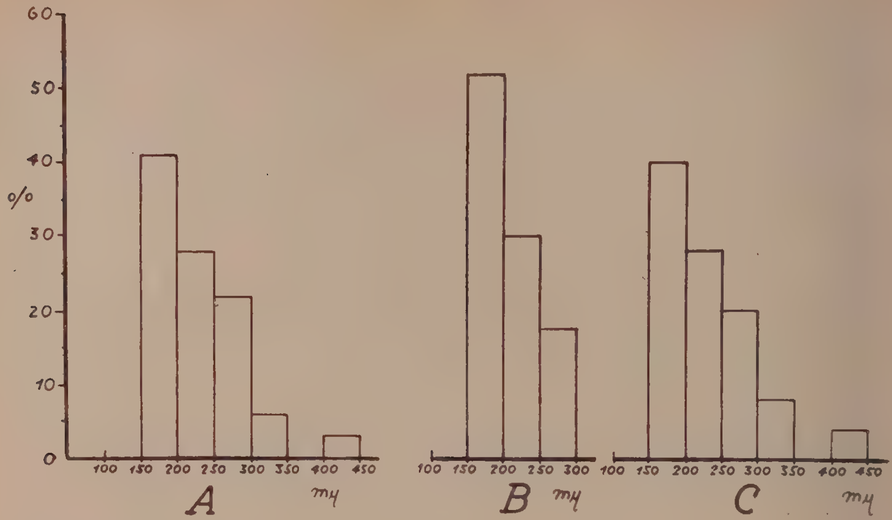

FIG. 9. Distribution curve of the diameters of elementary bodies of *Anaplasma*. A—Distribution of all 119 measurements; B—distribution of bodies within the marginal parasite; C—distribution of bodies dispersed throughout the erythrocyte.

certainly could not be observed with an optical microscope. The fewer larger ones are just at the limit of resolution of the microscope and could easily be mistaken with granular basophile material present in some normal erythrocytes and particularly in cases of hemolytic anemia.

These two facts are suggestive of the presence of smaller units of the parasite in addition to the marginal bodies shown by the optical microscope. The presence of these submicroscopic units might explain some of the clinical features of this disease, such as the persistence of the infective agent in the blood stream after the general symptoms of the disease have disappeared and the fact that the marginal bodies are at very low concentration or can not be ascertained with certainty.

These preliminary observations are also suggestive of a multiple division of the parasite instead of the generally accepted binary fission. The morphological entities of classical marginal bodies and the submicroscopic elementary bodies found with the electron microscope within the parasite or dispersed throughout the erythrocyte may probably represent stages of the life-cycle of the *Anaplasma* within the blood stream.

Summary. 1. Erythrocyte membranes from calves infected with *Anaplasma marginalis* were studied under the electron microscope. The classical single marginal body of the parasite appears to be constituted of a central undivided mass and peripheral elementary bodies of high electron density, 170 to 220 mμ in diameter. In some cases the whole mass of the parasite is divided into these small submicroscopic bodies. In other erythrocytes the elementary bodies are dispersed and have a tendency to grow up. Distribution curves on measurements of 119 elementary bodies are presented. 2. The presence of the submicroscopic forms of the parasite is discussed in the light of the clinical and hematological evolution of the disease, the multiplication and the probable life-cycle of the parasite within the blood stream.

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Effects of 4-Amino-N^{10}-Methyl-Pteroylglutamic Acid on the Extracellular Fluids of Rats.* (18743)

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During studies of the actions of folic acid antagonists in mice, rats, and dogs(1-3), it was noted that animals receiving fatal doses reduced their food and water intake, developed severe diarrhea, and appeared dehydrated at death. Such observations suggested that lethal effects might be related to alterations in body fluids. Accordingly, this report presents measurements of the changes produced in rat extracellular fluid by 4-amino-N^{10}-methyl-pteroylglutamic acid (aminomethyl-PGA).

Methods. The rats (Wistar) were equally divided by sex and weighed an average of 201 g. Those used for study of fluid volumes varied in weight from 148 to 259 g, a range in which plasma volume and "thiocyanate space" have been shown to be linear functions of body weight(4). Non-sterile injections of aminomethylPGA were given intraperitoneally in a dose of 100 mg/kg (fatal to most rats in 3 to 7 days)(3). Three or 4 days later, animals manifesting diarrhea, dehydration and weight loss of about 15% were sacrificed. Of the injected animals, 54% were sacrificed, 15% died and 31% survived. Heart blood was obtained under ether anesthesia and heparin-coated syringes were used when whole blood or plasma samples were required. Colorimetric procedures were employed for determination of calcium (method of Roe and Kahn), phosphorus (Fiske and SubbaRow), sugar (Folin-Wu), and creatinine (alkaline picrate method). Sodium and potassium were measured with the flame photometer(5). Nitrogen values were obtained by micro-Kjeldahl digestion and distillation. Total CO_2 was measured by the method of van Slyke and Neill. A modified iodometric method was used for chloride measurements (6). Plasma and circulating red cell volumes and "thiocyanate space" were determined simultaneously with sodium thiocyanate (20 mg/cc) and Evans Blue (1.2 mg/cc) given in a volume of 0.25 cc/100 g weight into the exposed saphenous vein of etherized animals. After a 5-minute interval, considered adequate for complete mixing(7), blood was drawn from the heart for determination of hematocrit and plasma concentrations of thiocyanate and Evans Blue(7) and calculation of circulating red cell volume. For balance studies, rats were maintained over wire screens in funnel bottom cages. To prevent scattering, ground food pellets (GLF Lab. Chow) were mixed with 10% by weight of peanut oil. Rats were maintained several days under these conditions before tests were begun.

Results. Table I summarizes results of the various analyses performed with rats intoxicated with aminomethylPGA. Plasma volume and "thiocyanate space" were reduced 18% and 17% respectively. Inasmuch as measures of "thiocyanate space" provide a useful estimate of extracellular fluid volume(8), these results indicate that intoxicated animals suffered significant losses of extracellular water.

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* This study was supported by a grant-in-aid from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

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TABLE I. Effects of AminomethylPGA and of Starvation on Extracellular Fluid of Rats.

		Control rats			Test rats			Difference of means	
Factor	Units	Mean	No. of tests	Stand. dev.	Mean	No. of tests	Stand. dev.	% change	Statistically significant‡
Effects of aminomethylPGA, 100 mg/kg, I.P.									
Thiocyanate space	cc/100 g†	28.	10	1.8	23.	13	1.8	–17	Yes
Plasma vol.	”	4.4	19	.6	3.6	22	.6	–18	”
Red cell ”	”	5.3	19	.8	5.6	22	1.4	+6	
Sodium (serum)	m Eq./1	144	6	1.2	144	5*	2.1		
Potassium (serum)	”	4.7	6	.8	4.5	5*	.4	–4	
Calcium (plasma)	mg/100 cc	5.5	6*	.6	3.7	7*	.5	–33	Yes
Phosphorus ”	”	4.1	6	.3	3.1	6	.3	–24	”
Chloride ”	m Eq./1	92	6*	8.7	96	6*	11	+4	
Total CO_2 ”	”	32	6*	2.9	30	6*	1.3	–6	
Protein (plasma)	g/100 cc	5.5	6*	.3	4.5	8*	.3	–18	Yes
N.P.N. ”	mg/100 cc	38	6*	3.7	36	8*	6.5	–5	
Creatinine ”	”	0	6		.6	6	.5		Yes
Sugar (blood)	”	89	6	4.5	94	6	45	+6	
Effects of complete starvation									
Thiocyanate space	cc/100 g†	27	12	3.7	24	15	2.1	–11	Yes
Plasma vol.	”	4.7	12	.6	4.1	15	.6	–13	”
Calcium (plasma)	mg/100 cc	4.2	6	.3	3.9	6	.2	–7	
Phosphorus ”	”	4	6	.4	3.6	6	.3	–10	
Protein ”	g/100 cc	6.3	6	.6	5.9	6*	1	–6	
Creatinine ”	mg/100 cc	0	6		0	6			

* Tests done on pooled samples.
† Expressed in g body wt at time of sacrifice.
‡ $P \leq .05$ (Fisher "t" test).

The full extent of the reduction in "thiocyanate space" may have been obscured by the coincident decrease in plasma protein concentration, since plasma proteins are known to bind thiocyanate ion(8,9). The loss of extracellular water was in keeping with the appearance of dehydration. Total circulating red cell volume did not change significantly in intoxicated rats, either when calculated in terms of the final weight (as in Table I) or original weight.

Among the blood electrolytes, plasma calcium and phosphorus values were respectively 33% and 24% lower in intoxicated rats than in controls. Serum sodium and potassium, as well as plasma chloride and total CO_2 concentrations were unaltered by aminomethylPGA. It should be noted that this maintenance of normal concentrations in a reduced volume represented a loss of these electrolytes. As shown in Table I, no detectable amounts of creatinine were found in control plasma samples (the method used did not give colorimetric readings at concentrations less than 0.06 mg %). However, the plasma of all but one rat intoxicated with aminomethylPGA contained measurable amounts. The mean value for whole blood sugar was essentially the same in both groups of rats, although poisoned animals showed extreme variations. Non-protein nitrogen remained unchanged.

Further experiments were performed to elucidate the mechanism by which the losses of protein and water occurred. Table II shows the effects of aminomethylPGA (100 mg/kg I.P.) on the weight, food consumption, water intake, urine volume, and urinary excretion of nitrogen and chloride of 12 rats. Two died on the fourth day, 8 others died by the seventh day, and 2 survived. It is apparent that increased excretion of water and nitrogen did not occur. However, the food consumption was almost completely eliminated by the fourth day, even in those rats which survived the effects of aminomethylPGA. The water intake, urine volume, and urine nitrogen and chloride excretion were somewhat reduced after administration of aminomethylPGA. In a second series of rats, the voluntary food consumption was observed on 2 control days

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TABLE II. Effects of AminomethylPGA and Starvation on Metabolism of Rats.

	Control (mean of 2 days)	Test days			
		1	2	3	4
Effects of aminomethylPGA (100 mg/kg I.P.) on 12 rats*					
Food consumed (g/rat/day)	11.3	7.4	5.5	1.8	.8
Wt (g)	213	213	210	196	191
Water intake (cc/rat/day)	19.7	13.2	12.2	10.9	11.8
Urine vol. ”	2.3	2.1	1.6	3	1.9
” N (mg/rat/day)	78	71	53	57	53
” chloride (m Eq/rat/day)	.039	.059	.022	.017	.015
Effects of progressive food restriction on 12 rats*					
Food supplied (g/rat/day)	11	7.2	5.4	1.8	.8
Wt (g)	204	205	199	192	185
Water intake (cc/rat/day)	20	18.6	14.6	11.2	9.4
Urine vol. ”	2.3	2.1	1.9	2.1	1.9

* Rats studied in pairs.

and thereafter restricted to amounts proportional to the food intake *ad libitum* exhibited by the intoxicated rats. These rats manifested essentially the same effects on weight, water intake and urine volume as the rats given aminomethylPGA (Table II).

Inasmuch as anorexia was a predominant feature of aminomethylPGA-intoxication, it seemed necessary to assess the role of starvation in the production of the biochemical changes observed in poisoned animals. For this purpose rats were completely deprived of food, though permitted free access to water, for a 3-day period, sufficient to reduce their weight by 15%. This weight reduction, although comparable to that found in intoxicated animals at the time of sacrifice, was not accompanied by overt signs of dehydration. Those factors which had been altered by administration of aminomethylPGA were determined in these starved animals (Table I). The "thiocyanate space," plasma volume, and plasma protein, calcium and phosphorus were all reduced by starvation but only in the case of the fluid volumes were the reductions statistically significant. Plasma volume was reduced 13% and "thiocyanate space" 11%, compared to the reductions of 18% and 17% respectively, which followed administration of aminomethylPGA. Plasma creatinine was not elevated by starvation. An incidental observation in these starved rats was an elevation of total red cell volume, a finding not seen in poisoned animals and for which no explanation was apparent.

An attempt was made to alter the toxicity of aminomethylPGA by changes in the state of hydration of animals. Mice were used, since the lethal response of rats to single doses of aminomethylPGA was previously shown to be erratic(3). Daily intraperitoneal administration of 2 cc of isotonic saline solution (approximately half the daily water intake of control mice) failed to lower the mortality of an LD_{50} of aminomethylPGA. Similarly, abstinence from water for 26 hours prior to injection of aminomethylPGA did not increase the toxicity of an approximate LD_{10}.

Discussion. Rats poisoned with aminomethylPGA manifested weight loss, reduced consumption of food and water, and reduced urinary output. Of these effects, only the reduced food consumption seemed due primarily to actions of the drug. The restriction in food intake was noted in previous studies(3) but its extent not fully appreciated. Even rats with little other evidence of intoxication refused food almost completely. This effect may be related to the intestinal lesions produced by this compound(3).

The loss of plasma protein and at least part of the loss of body water may also be ascribed to the intestinal lesion. As previously described(1-3), anti-folic acid agents cause desquamation of the intestinal mucosa and production of an ulcerative enteritis. In many animals extravasation of plasma into the intestinal lumen was observed microscopically and significant amounts of protein and water were undoubtedly lost by this means. Certainly urinary excretion cannot account for the losses of water and protein. Although starvation was productive of a mild dehydration, the results stated herein, together with the reports of others(10,11) indicate that the restricted food consumption associated with intoxication by aminomethylPGA cannot account for the observed protein and water losses.

The mechanism by which aminomethylPGA elevated plasma creatinine and depressed plasma calcium and phosphorus concentrations is obscure. Starvation did not duplicate these effects. Probably part of the lost calcium was that combined with the plasma protein extruded into the intestine.

It is difficult to evaluate the significance of the observed biochemical changes. Both the starvation and the dehydration induced by administration of aminomethylPGA seem compatible with life. The failure of forced hydration to prevent death in intoxicated mice further minimizes the significance of the dehydration. The loss of plasma protein may be of greater importance. It may be calculated from the observed changes in body weight, plasma volume and plasma protein concentration that approximately 40% of the initial plasma protein was lost during the period of intoxication with this drug.

It is of interest to compare the effects of the present folic acid antagonist with those of the nitrogen mustards. The actions of both groups of compounds are characterized by anorexia, weight loss, bone marrow damage, diarrhea associated with intestinal lesions, and delayed death. Previous investigators(12,13), studying the effects of the mustards in dogs, found that fatally poisoned animals lost significant amounts of body water and protein, principally from the intestinal tract. However, as concluded in the present study with aminomethylPGA, these changes were not considered sufficient to account for the lethal actions of nitrogen mustards.

Summary. Administration of 4-amino-N^{10}-methyl-pteroylglutamic acid to rats caused reduction of food consumption, extracellular fluid volume, plasma volume and plasma concentrations of protein, calcium, and phosphorus. Plasma creatinine concentration was elevated. Starvation alone did not duplicate the effects of this compound. Increased urinary excretion of nitrogen and water did not occur. The loss of body water and plasma protein appears to be the consequence of a previously described intestinal lesion produced by anti-folic acid agents. The relation between these biochemical changes and the mechanism of lethal action remains obscure.

The author is indebted to Dr. Oscar Bodansky for the flame photometric determinations of sodium and potassium and to Dr. Frederick S. Philips for advice and criticism. Supplies of 4-amino-N^{10}-methyl-pteroylglutamic acid were furnished by investigators of the Lederle Laboratories and the Calco Chemical Division, American Cyanamid Co.

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A Spontaneous Epizootic of Mouse Encephalomyelitis.* (18744)

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Two main groups of mouse encephalomyelitis viruses were described by Theiler(1,2). The TO type virus produces flaccid paralysis,

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* Aided by a grant from The National Foundation for Infantile Paralysis.

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has a long incubation period (7-30 days), and requires a relatively high concentration of virus to infect (dilutions of 10^{-3} to 10^{-5}). The GD-FA types produce encephalomyelitis, usually without paralysis, have a short incubation period (2-9 days), and infect in relatively high dilutions (10^{-6} to 10^{-8}). According to Olitsky(3) the viruses are antigenically related but not identical. Spontaneous paralysis due to the TO type has been reported many times(4), but the total incidence is only in the neighborhood of once in every 10,000 mice. Occurrence of the encephalitic-type of disease has been reported a number of times following intracerebral injections of various materials. Theiler(2) isolated encephalitic virus from mice which became ill following inoculation with yellow fever virus. Melnick and Riordan(4) reported seven instances in which the disease followed intracerebral inoculation of Lansing-type polio virus or of noninfectious material. In one instance the disease occurred spontaneously without previous inoculation. This appears to be the only reported case of spontaneous occurrence. To our knowledge no epizootic due to either type virus has ever been reported.

Circumstances under which the epizootic occurred. The epizootic to be described occurred during the course of experiments on the passive transfer of immunity to Lansing virus from immune mothers to their own, or to foster, young(5). Female mice were immunized intraperitoneally or subcutaneously with Lansing virus or with normal mouse brain-cord suspensions. In the particular experiment concerned, the mother mice were vaccinated during pregnancy. At birth, half the young were left with their own mothers, and half transferred to a mother of the opposite group, that is from immune to control mothers, or vice versa. The young were weaned at 28 days. The object of this experiment was to determine the duration of the passive immunity, and challenge injections were to be made at 2, 4, 6, and 8 weeks after weaning. It was during the period of waiting before challenge that the epizootic deaths occurred in most groups.

At detection of pregnancy the mothers were placed in glass jars with sawdust bedding. The food, fox checkers, was placed in the bottom of the jars. Water was supplied by gravity feed bottles with glass tubing. At weaning, the mothers were removed and the young left in the jars. Additional sawdust bedding was added as necessary, but in most cases the jars were not cleaned out during the experiment.

The Epizootic. Occasional deaths had occurred among the mothers in the experimental breeding cages, during November, December, and January. During January a few deaths occurred in some groups of uninoculated young, but no nervous symptoms were noted. Early in February the young began to die off rapidly, and nervous symptoms were noted in about 50% of the mice which died. About 150 of a total of 240 uninoculated young mice died during the acute stage of the epizootic. The peak was reached February 14th when 49 mice died. Scattered deaths occurred during late February and March.

There was no relation of the outbreak to the exposure of the mothers to Lansing virus. The control young, whose mothers had no contact with this virus, had a death rate of 57% in the epizootic. The "immune" young, born of and fed by mothers immunized by active Lansing virus, had a death rate of 60%. The young transferred from control to immunized mothers had a mortality rate of 67%, and those from immunized to control mothers, one of 54%. There was apparently no relationship of the epizootic to the age of the young mice. The different age groups had mortality rates as follows: 60-66 days, 80%; 50-59 days, 57%; 40-49 days, 30%; 30-39 days, 81%. Only one group of unweaned mice was concerned: of 6 mice, 19 days of age, 3 died with typical symptoms. The outbreak was confined to the mice in the experimental room. No deaths occurred in the normal breeding room. In the experimental room all jars were

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4. Melnick, J. L., and Riordan, J. T., *J. Immunol.*, 1947, v57, 331.

5. Thompson, R., and Meyers, F. P., *Am. J. Hyg.*, 1950, v52, 213.

affected, but the majority of deaths at the height of the epizootic occurred in one part of the room.

Isolation of virus. At the height of the epizootic on February 14th, 6 mice with typical nervous symptoms were killed. A 20% emulsion of the pooled brains and cords was inoculated intracerebrally into 10 normal 28-day-old mice. All of these died within 2 days with typical nervous symptoms. Two other consecutive passages were made with similar results. Cultures on blood plates and in broth, of the brain-cord emulsions used for passage, were consistently negative. No evidence of the growth of bacteria or of pleuropneumonia organisms was seen. A 5% broth emulsion of the second-passage virus after passage through a Seitz filter produced typical symptoms and death in 5 of 5 mice.

Symptoms were similar in the spontaneously-infected mice and in the experimentally-infected. Death without symptoms was somewhat more frequent among the spontaneously-infected. Paralysis was never seen in the spontaneous cases while, as noted below, it was occasionally seen in the experimentally-inoculated mice. Often the earliest symptom was a pulling of the head to the left or right, with a tendency to circling in the same direction. The circling increased with the progress of the disease and with stimulation by noise. As the disease progressed, the circling became a pivoting with the base of the tail as the center of the circle. Frequently this progressed to a rolling in the same direction. Sometimes the rolling was spontaneous, but most frequently was produced by noise stimulation. Some mice showed merely excitability and generalized convulsions on noise stimulation. Some showed marked generalized tremors, and in others great respiratory difficulty was the only symptom. Occasionally the front paws were clenched up under the lower jaw. Weakness or flaccid paralysis of the hind legs occurred occasionally in some of the experimental mice.

Preliminary titration studies indicated that the virus isolated had a 50% end-point dilution titer of 10^{-6} to 10^{-7}. The short incubation period, the titer, and the symptoms all indicated a virus of the encephalitic type. Careful comparative studies were made of the new virus and of the GD VII virus which had been passed for some months in the laboratory. In view of the possibility that this GD VII virus (designated GD VII A) had been contaminated while in our laboratory, we obtained also another GD VII passage from Dr. Theiler, and this virus (designated GD VII B) was used in the comparisons. The three viruses were similar in all respects. Injected intracerebrally into 28-day-old mice they all produced the symptoms described above.† The incubation period was 2 to 9 days in all cases. The epizootic virus had a 50% end-point dilution titer of 10^{-6} to 10^{-7}, as compared with the 10^{-5} to 10^{-6} range of the two stock viruses. Both the epizootic virus and the GD VII A virus produced typical disease on the intraperitoneal or intranasal inoculation of undiluted 10% emulsion in 28-day-old mice. The GD VII B strain was not tested in this respect. All 3 viruses on intracerebral injection into 30-day-old hamsters produced encephalitic or paralytic disease. No symptoms were produced on intracerebral inoculation of young guinea pigs by either the epizootic or the GD VII A virus.

To determine the antigenic relationship of the GD and new viruses, 110 mice from 28 to 34 days of age were divided into 3 groups with

TABLE I. Cross-Immunity Between GD VII Virus and the Virus Isolated from the Epizootic.

Virus used to immunize	Virus used for challenge	No. of mice challenged	No. of mice surviving	% of mice surviving
GD VII B	GD VII B	19	11	58
,,	Epizootic	19	9	47
Epizootic	GD VII B	19	13	68
,,	Epizootic	19	6	31
0	GD VII B	17	1	6
0	Epizootic	17	1	6

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† Extensive pathologic studies could not be done, but Dr. Karl Neuberger of our department of pathology examined sections of several of the spontaneously-affected mice. Microscopic changes were surprisingly scanty. Lymphocytic infiltration was present around several small vessels, especially in the medulla. There was a tendency to fragmentation of the nuclei of these infiltrates and a patchy disintegration of the nerve cells with glial proliferation.

TABLE II. Cross-Neutralization Between GD VII Virus and the Virus Isolated from the Epizootic.

Virus used to produce serum	Dilution of serum	Virus used for inoculation	No. of mice inoculated	No. of mice surviving	% of mice surviving	Normal serum controls* No. of mice inoculated	No. of mice surviving	% of mice surviving
GD VII B	500	GD VII B	14	12	86	10	0	0
	1000	”	14	6	43	17	0	0
	100	Epizootic	10	10	100	10	0	0
	500	”	10	9	90	10	2	20
	1000	”	11	1	9	—	—	—
	1500	”	11	1	9	—	—	—
Epizootic	500	Epizootic	12	8	67	8	2	25
	1000	”	12	4	33	8	3	37
	10	GD VII B	7	7	100	7	3	43
	500	”	10	7	70	10	0	0
	1000	”	17	10	59	17	0	0
	1500	”	12	1	8	—	—	—

* Inoculated at the same time as the corresponding experimental group with pool virus from same vial. The normal serum dilution used was the same as the immune serum dilution.

litter mates divided evenly between the groups. Group 1 was immunized against the new virus; Group 2 against the GD VII virus; and Group 3 was left as control. The immunizations consisted of intraperitoneal injections of the virus suspensions at 3-day intervals over a 5-week period, beginning with a 0.1 cc injection of a 1:10,000 dilution, and building up to a 0.3 cc injection of a 1:10 dilution. Immune and control mice were challenged by an intracerebral injection of 0.03 cc of a 10^{-4} dilution of each of the viruses. The results are shown in Table I.

Each virus produced a considerable degree of immunity against the other. There was no indication of any significantly greater degree of immunity against the homologous than against the heterologous strain. The epizootic virus would appear to be antigenically identical or very similar to the GD VII strain. Since the cross-resistance could conceivably be due to virus interference, rather than to antigenic relationship, it seemed necessary to perform cross-neutralization tests. Using the same methods and dosages as in the active immunity experiment, one group of 32 mice was immunized to GD VII B virus, and another group of 32 mice to the epizootic virus. Both groups were bled by decapitation on the 10th day after the last injection. The serums from the animals in each group were pooled, distributed in vials in 1 cc amounts, and preserved at —70°C until used. Serum from normal animals of corresponding age was similarly prepared and preserved. For neutralization tests serial dilutions of the serums were mixed with equal quantities of a $10^{-3.5}$ dilution of the virus to be tested, and 0.03 cc of the mixture inoculated intracerebrally into etherized 28-day-old mice. The viruses were from previously prepared and titrated pools kept at —70°C. The results of the neutralization experiments are shown in Table II. These results confirm the findings of the active cross-immunity experiment, inasmuch as serum protected against the other virus at least equally as well as against the virus used to produce it.

Discussion. We have no explanation as to why this unusual epizootic occurred at the time it did. The epizootic was apparently due to a virus identical or almost identical to the GD VII virus. Whether the virus originated from the GD VII strain being used in the laboratory, or whether it arose from our mouse colony or from wild mice which were present in the laboratory at times, cannot be determined. Spontaneous paralysis had occurred in the mouse colony on several occasions, and a virus of the TO type had been isolated on 3 separate occasions several years ago. No encephalitic-type virus had ever been isolated previously from this colony. The rather unsanitary conditions in which the experimental mice were kept may have been a factor. We can only speculate as to whether the series of injections of brain suspensions into the mother mice played any role. In retrospect, it would have been desirable to make more than one isolation of virus from the affected mice, but the great similarity of the disease in the spontaneously-affected and the experimentally-infected mice would seem to be a good indication that the virus isolated was actually responsible for the epizootic.

Summary. A spontaneous epizootic of encephalomyelitis which killed 62% of 240 uninoculated mice is described. A virus was isolated which resembles GD VII virus in its properties, and which is antigenically identical or very similar to the GD VII virus.

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Detection of Phosphatase Activity in Polarized Light Following Glycerophosphate Incubation.* (18745)

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The differences in the exact localization of phosphatase activity such as reported in the literature are due to several factors. Among these is the fact that in the Gomori technic of histochemical detection(1), the white precipitate formed is transformed into a black mass by the cobalt nitrate-ammonium sulfide treatment. Unfortunately such a treatment produces also some artificial blackening part of which has already been recognized, such as that of the ground substance of the cartilage. On the other hand, in the black areas of phosphatase activity, it is possible that some local changes in the amount and position of the precipitate might take place during the transformation of lead or calcium phosphate into sulfide.

In order to control these two factors of error, one might try and examine the initial precipitate, a thing which is made easy by using polarized light and an environment which will not reduce the anisotropism of the mineral salts.

Material and methods. Rat and hamster tissues were fixed in 80% alcohol or cold acetone. The tissues were embedded routinely in paraffin or low viscosity celloidin, according to a technic described previously(2). Sections of already mineralized material such as bones and teeth were treated with a buffer decalcifying solution of formic acid and sodium citrate at pH 4.9 according to Greep, Fischer, and Morse(3). By treating 10 mμ sections instead of blocks, the time in that solution was reduced from a matter of days to a maximum of 15 minutes. Incubation was carried out at 37°C in Gomori's solutions of sodium alphaglycerophosphate at pH 5.0 and pH 9.4 for acid and alkaline phosphatase respectively, and also other substrates such as glucose-1-phosphate, adenylic acid, ribonucleic acid, desoxyribonucleic acid and sodium desoxyribonucleate.

Following 3 hours of incubation, the sections were brought to room temperature and dehydrated in graded alcohols and finally mounted with a coverslip in 5% celloidin in alcohol-ether. The controls were similarly treated and all preparations had to be thoroughly dry before examination. In examining these preparations regular polarizing equipment has been used but in comparison, it has been possible to do just as well with a simple addition to a monocular microscope of 2 pieces of Polaroid material of type JG30FAP commonly used in antiglare goggles. These pieces are cut to appropriate sizes to fit the filter slot at the base of the condenser (polarizer) and the inside of the eyepiece (analyser) where the last one rests on the metal insert midway between the top and the bottom lenses. With this equipment, a red extinction of the field is obtained when the

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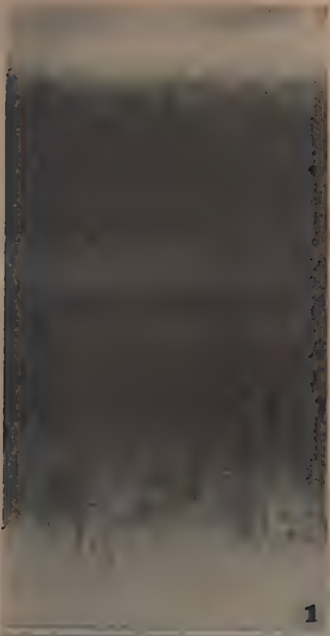

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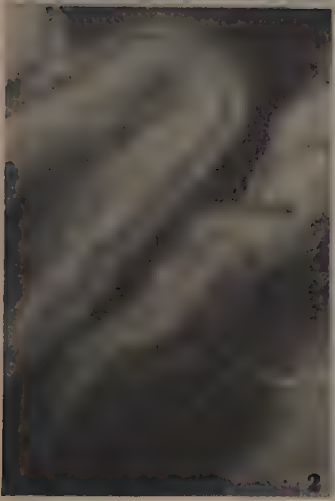

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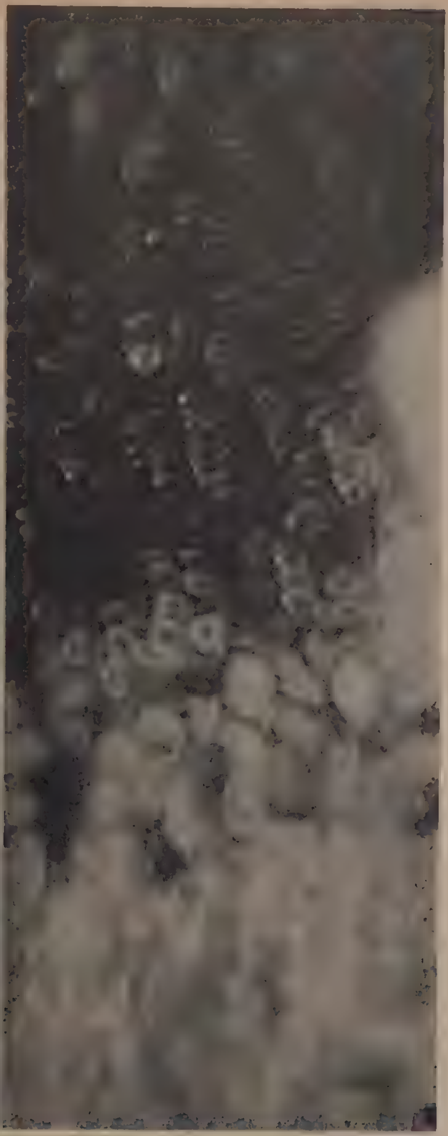

FIG. 1. First molar, upper jaw of an 8-day-old rat. Alkaline phosphatase after demineralization; glycerophosphate, 3 hr. ×315. From the top down can be seen the stratum intermedium of the enamel organ (white), the ameloblasts (dark), the enamel and dentine, the odontoblasts (dark) and the proximal region of the pulp (white).

FIG. 2. Villi of the ileum of an 8-day-old rat. Alkaline phosphatase, 3 hr. ×368. The epithelial cells show an extranuclear reaction (white) progressively increasing from the base towards the tip.

FIG. 3. Epiphyseal plate of the humerus of a 6-day-old hamster. Alkaline phosphatase after demineralization. 3 hr, glycerophosphate. ×315. The hypertrophic cells of the cartilage show an increasingly intense reaction. The bone spicules (lower part of the picture) are intensely positive as well as ''Ranvier's notch'' (in the middle of the picture, extreme right).

polarizing planes are crossed and the precipitates show in the complimentary yellow-green color.

By a procedure similar to that of Dempsey, Wislocki, and Singer(4) quantitative estimates of the amount of precipitate have also been obtained by comparison of the light absorption power of the precipitate of different areas in white light as the section is projected on the ground glass of a photographic apparatus and the light absorption is measured under comparable conditions with a sensitive densitometer. In undecalcified material, the natural mineral deposits also show with great detail, under polarized light in conditions such as mentioned above.

Observations. Part of the results will be reported elsewhere along with other studies of the phenomenon of mineralization of bones and teeth. For the present purpose, the attached pictures demonstrate the following facts:

Against the dark background, the areas of phosphate precipitates are seen in great detail. In the developing tooth (Fig. 1) the alkaline phosphatase reaction is visible behind the ameloblasts and also behind the odontoblasts. Thin lines of precipitate are visible between the distal ends of these 2 groups of cells. The substance of the dentine and of the enamel are both negative.

In the villi of the intestinal epithelium (Fig. 2) the reaction is present at the apical portion of the cell and it increases in intensity from the base to the tip of the villi. The mucosal glands are negative. In the areas of lesser activity in the villar epithelium, it is possible to see that the reaction is truly a cytoplasmic phenomenon.

At the epiphyseal plate of the long bones (Fig. 3), the phosphatase activity is present in the enlarged cells of the cartilage, over the new bone spicules and on the right hand side of the picture, over this terminal zone of the periosteum known as "Ranvier's notch." In the cells of the cartilage where the reaction is minimal, it appears to be definitely limited to the cytoplasma.

Comments. As compared to the localizations reported in the literature, several of the preceding descriptions are at variance. When the standard cobalt nitrate-ammonium sulfide treatment was applied to preparations similar to those described above, black precipitates were visible in other areas, notably over the nuclei of cells close to sites of phosphatase indicating possible displacement and adsorption of the precipitate as a result of this operation. Other areas were black in control preparations incubated without glycerophosphate. Such were to variable extents, the organic matrix of the dentine, enamel, bone and cartilage and the keratinized portion of the skin. All these areas were obviously negative in polarized light. On the other hand, certain tissues are known to have anisotropic properties on account of their chemical composition or simply on the basis of their physical arrangement. These were negligible in the preparations studied so far but the possibility of artefact must always be searched for in control slides.

In areas of actual precipitate, details are greatest in border regions where the phosphatase activity is minimal. They become blurred in areas of maximal deposition of calcium or lead salt (Fig. 3). Preliminary studies with the present technic demonstrate the following facts: (1) phosphatase activity in growing bones and teeth is localized to areas which are not necessarily those of mineral deposition (stratum intermedium of enamel organ, pulp of the teeth, Ranvier's notch in long bones); (2) phosphatase activity originates in the cytoplasma of cells; (3) it increases with the differentiation of the cell (enlarged cartilage cells and epithelium of the villi).

Summary. A method of detection of phosphatase activity is described whereby some artefacts of diffusion, adsorption and nonspecific sulfide blackening are dealt with by examination of the original calcium or lead phosphate precipitates in polarized light. This technic demonstrates also the presence of natural mineral deposits in undecalcified material. Comparable estimates of the amount of salt deposited is made with the help of a densitometer.

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Toxicology of Podophyllin.* (18746)

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When the colchicine-like effects of podophyllin were demonstrated(1,2), it was anticipated that the resin of podophyllin would be investigated in the treatment of cancer of the skin. Subsequent exhibitions of selective damaging effects of podophyllin and podophyllotoxin on mouse tumor cells *in vitro*, confirmed by *in vivo* studies in tumor bearing mice(3-5) stimulated the initiation of several clinical trials now in progress(6-8). Phillips, Chenowith, and Hunt(9) found the LD_{50} of podophyllotoxin to be 33 mg/kg for mice and 15 mg/kg for rats. Greenspan and Leiter(10) reported the maximum tolerated single dose of podophyllotoxin in mice to be approximately 30 μg/g.

The purposes of this investigation were: 1. To compare the toxicity of podophyllin with that of colchicine in mice. 2. To obtain additional toxicity data in rats on podophyllin and its components as a prelude to the clinical use of podophyllin in the treatment of cutaneous carcinoma(6). Podophyllin U.S.P. is a complex resinous mixture which contains: podophyllotoxin, the component principally responsible for the drug's curative effect on condyloma acuminatum(2,11,12); quercetin; and 2 recently isolated crystalline components, designated as alpha-peltatin(13) and beta-peltatin(14). Inasmuch as alpha-peltatin and beta-peltatin were unobtainable, our study was limited to podophyllin; podophyllotoxin and its 2 products of alkaline hydrolysis, namely, (1) picropodophyllin and (2) podophyllic acid; quercetin; and depodophyllotoxinized resin. Detailed descriptions of the aforementioned 6 materials are recorded elsewhere(12,15).

Method. Material for parenteral administration was prepared by dissolving each test substance, with the exception of picropodophyllin, in absolute alcohol. One cc of a one or 10% alcoholic solution of the test substance was added to 9 cc of a 10% gum acacia suspension or to a 0.5% methocel suspension. Picropodophyllin was suspended in water with methocel. Test materials for oral administra-

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TABLE I. Median Lethal Single Dose in Adult Albino Farm Mice, Averaging 20 g, of Drugs Tested, by Route of Administration.

Drug	No. of mice	Route	LD_{50}, mg/kg	S.E. LD_{50}
Colchicine	106	Subcut.	3.8	.34
Podophyllotoxin	117		24.6	.42
	150	Oral	90	2.2
Podophyllin	140	Subcut.	58	1.7
	130	Oral	68	4.8
Quercetin derived from podophyllin	158	Subcut.	97	3.7
	197	Oral	161	3.9
Quercetin derived from quercetron	218	Subcut.	99	2.5
	281	Oral	159	2.7
Picropodophyllin	188	Intraper.	280	22.7
Depodophyllotoxinized resin	171	Subcut.	522	11.1
	199	Oral	625	15.9
Podophyllic acid	167	Subcut.	700	35.3
	136	Oral	899	15.3

TABLE II. Median Lethal Single Dose in Young and Adult Rats of Podophyllin and Podophyllotoxin Administered Subcutaneously.

		Young rats		Adult rats	
Drug	No. of animals	LD_{50} mg/kg	S.E. LD_{50}	LD_{50} mg/kg	S.E. LD_{50}
Podophyllin	74	18	1.4		
	159			24	1.1
Podophyllotoxin	84	8	.81		
	85			22	1.2

tion were incorporated in gum acacia or methocel suspensions. Autopsies were performed immediately after death. The following tissues were examined: skin, heart, lung, trachea, thyroid, parathyroid, tongue, stomach, small and large intestines, gonads, spleen, pancreas, brain, striated muscle, bone with marrow, and eye.

In Table I are summarized median lethal doses of substances tested for adult mice. The method used for obtaining the LD_{50} values and their standard errors was that of Litchfield and Fertig(16). In Table II are summarized comparative toxicity data on podophyllin and podophyllotoxin for young, recently weaned rats, averaging 28 g, and adult rats, averaging 150 g of the McCollum strain. Considered to be of interest were the following observations on symptomatology and pathology.

Podophyllin. Death occurred usually within 15 to 18 hours after parenteral administration or ingestion of fatal doses of podophyllin. Diarrhea, rapid and labored respiration and a dragging gait with hyperextended posterior extremities were observed in the majority of the animals during the 8 hours after dosing. At 9 hours there was a period of excitation, during which the animals ran wildly, and which was climaxed by spastic convulsions. This was followed by the terminal period, manifested by flaccidity, a dragging gait and slow, shallow, labored respirations. Microscopic studies were performed on 15 of the rats. The most constant finding was an acute enteritis. There were increased numbers of polymorphonuclear leukocytes in the submucosa of both small and large intestine and a striking breaking up of their nuclei so that small masses of deeply staining chromatin were found in profusion. Another prominent change was necrosis of the lymphoid tissue beneath the mucosa, usually affecting the central portion; again there were numerous aggregations of bits of chromatin. There was no apparent change in the mucosal epithelial cells; there did seem to be an increase in mitotic figures in their nuclei. Similar evidence of nuclear distintegration was present in lymphoid follicles of the spleen where again were found bits of chromatin. Increased mitotic activity was present in the epithelium of the tongue. There were no arrested mitoses in the skin.

Podophyllotoxin. Eight hours after administration of fatal doses of podophyllotoxin the animals were flaccid, their respirations were slow, shallow and labored. The terminal stage, in which they were somnolent or unconscious, lasted 4 to 7 hours, and death always occurred within 12 to 15 hours. Microscopically, 7 rats which had received a single dose of the toxin in this group were examined; all appeared normal. Four rats that had survived an initial LD_{50} dose were injected 2 weeks later with a fatal dose of podophyllotoxin; they showed the same changes in the intestine which the podophyllin treated rats had exhibited and they will not be detailed again. The spleens were also affected in the 4 rats that had received the 2 doses.

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TABLE III. Cumulative Effects of Podophyllin and Podophyllotoxin.

		Individual dose			
	No. of rats	Fraction of LD_{50}	mg/kg	No. per day	No. of days
Podophyllin	8	1/12	1.9	1	15, 105, 105, 105, 105, 105, 105, 105
	8	1/6	3.8	1	7, 7, 14, 15, 26, 31, 56, 105
	10	,,	3.8	2	4, 4, 5, 5, 5, 6, 6, 6, 6, 9
	10	,,	3.8	3	3, 3, 3, 3, 3, 3, 3, 4, 4, 4
	10	,,	3.8	4	2, 2, 3, 3, 3, 3, 3, 3, 3, 4
Podophyllotoxin	5	1/10	2.2	1	5, 12, 38, 63, 105
	7	1/5	4.4	1	12, 15, 16, 50, 57, 63, 105
	10	,,	4.4	2	2, 2, 4, 4, 6, 6, 6, 6, 30, 30
	10	,,	4.4	3	3, 3, 3, 4, 4, 5, 5, 5, 6, 6
	10	,,	4.4	4	1, 2, 2, 2, 3, 3, 3, 3, 3, 3

Picropodophyllin. Terminal signs were rapid respiration and tremors. Gait was normal until the terminal stage when flaccidity developed. Death occurred within 16 to 18 hours. No histologic observations were made.

Podophyllic acid. Death occurred in 17 to 18 hours and the terminal signs were similar to those produced by podophyllin. No histologic observations were made.

Quercetin. The terminal signs of poisoning from quercetin differed from those of podophyllin and podophyllotoxin. Within half an hour after injection the animals exhibited an abnormal gait in which there was a rapid swimming motion of the forepaws, following which all locomotion was accomplished by the posterior extremities with which they propelled themselves. During the next half hour their respirations became labored and they lay in a somnolent state on one side occasionally attempting to move. There was apparently some pruritus as all scratched their sides. Finally in an hour they became relaxed, flaccid, and unable to drag themselves forward. Following a period of 2 hours of rapid, deep breathing their respirations became shallow and slow and death occurred at 4 hours after the injections. A group of 10 adult rats, fatally poisoned by single injections of quercetin, was examined microscopically; no changes to account for death were found. The rapid lethal action of the drug may account for the absence of detectable alterations in the postmortem tissues.

Depodophyllotoxinized resin. The animals that died exhibited the following signs: fine tremors and a dragging, clumsy gait with their hind extremities hyperextended. Preceding death there were flaccidity, general weakness and slow, labored respirations. Death occurred usually 6 to 9 hours after injection of fatal doses. Microscopic examinations of 11 adult rats, fatally poisoned by single injections of depodophyllotoxinized resin, showed the intestinal damage we have already described in rats that died following injections of podophyllin. The spleen also showed nuclear fragmentation.

Colchicine. Thirteen rats fatally poisoned with single injections of colchicine were studied microscopically. Changes similar to those already described in rats poisoned with podophyllin were present in the intestine and spleen. In addition there was a marked increase in mitotic figures in the epitheliums of a number of tissues, namely, skin, tongue, esophagus, fore and hind stomach, intestine and gonad.

Cumulative effects of podophyllin and podophyllotoxin. The experiments summarized in Table III were designed to determine whether cumulative effects would result from the administration of daily, fractional, sublethal doses. When podophyllin and podophyllotoxin were administered subcutaneously in single daily doses of fractions of the LD_{50}, cumulative effects were produced in the majority only after prolonged periods. Cumulative effects were produced in shorter periods with similar single doses administered 2, 3 and 4 times a day.

Summary. 1. The median lethal doses of podophyllin, podophyllotoxin, picropodophyllin, podophyllic acid, quercetin from podophyllin, quercetin from quercetron, podophyllotoxinized resin and colchicine were determined in adult mice. In this species podophyllin and podophyllotoxin respectively are approximately 1/15 and 1/6 as toxic as colchicine. 2. A comparison of the median lethal doses of podophyllin and podophyllotoxin in young and adult rats demonstrated a greater degree of toxicity for young rats. 3. Cumulative toxic effects were produced in rats by administering podophyllin and podophyllotoxin multiple times a day in individual doses that were in the range of ⅙ and ⅕ of the LD_{50}. 4. Observations on the symptomatology and/or pathologic alterations of rats poisoned with podophyllin, podophyllotoxin, podophyllic acid, picropodophyllin, quercetin and depodophyllotoxinized resin and colchicine are presented. 5. In animals that succumbed to subcutaneous injections of colchicine there was an accumulation of arrested mitoses in the skin (positive Dustin reaction); in animals killed by subcutaneous injections of podophyllin the Dustin reaction was negative as there were no epidermal changes.

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Effect of Semliki Forest Virus on Rabbit Fibroma.* (18747)

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Certain neurotropic viruses have been shown to parasitize transplantable mouse tumors and cause necrosis of the tumors(1-3). Similar antagonism of a transplantable lymphoid tumor of chickens by Russian spring-summer and other neutrotropic viruses has been reported(4). It has also been demonstrated that Virus III causes suppression or early regression of the rabbit fibroma(5). The inhibiting action was demonstrated only when Virus III was inoculated at the same site as the fibroma virus. The present paper describes the results of a study of Semliki Forest virus (SFV) in tissue cultures of normal and tumor cells and the destructive effect of this agent on virus-induced rabbit fibromas.

Methods. Viruses. SFV was prepared as 20% suspensions of infected mouse brain. The intracerebral LD_{50} titer in 10 g mice varied from $10^{-7.5}$ to $10^{-9.0}$. Fibroma virus was obtained from Dr. M. H. D. Smith. This strain of virus was isolated by Dr. R. E. Shope and is similar in its characteristics to the original OA strain. Fibromas were produced on the abdomens of groups of New Zealand white rabbits by injecting 0.5 cc of a 10% extract of testicular fibroma tissue intradermally into 6 to 8 sites. Simultaneously, or at various intervals thereafter, 1.25 ml of a 20% SFV extract was injected intramuscularly. The course of fibroma growth was observed daily or every second day and the diameter and height of each growth was measured and recorded. The volume of each growth was estimated in cubic centimeters (cm^3) by multiplying the height $\times$ π radius^2. Weighed portions of fibromas were extracted, centrifuged and titrated intradermally on the abdomens of normal domestic rabbits, using 3 to 4 inoculations for each dilution and a 0.10 cc inoculum. The titer of a given extract was recorded as the Infectious $Dose_{50}$ (ID_{50}) (6). When fibroma virus titrations were made with extracts of fibroma that contained

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* This investigation was supported by a research grant from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

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2. Moore, A. E., *Cancer,* 1949, v2, 525.

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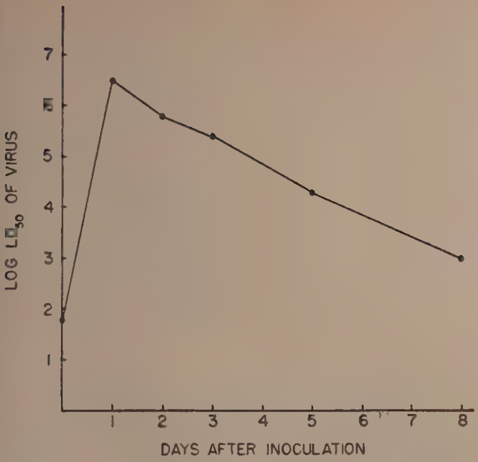

FIG. 1. Multiplication of SFV in tissue culture of chick embryo brain.

SFV as well as fibroma virus, SFV rabbit antiserum (that protected against 10,000 LD_{50} SFV virus) was added to the extracts. These mixtures were incubated 1 hour at room temperature before titration by the intracutaneous method in rabbits.

Tissue culture technic. Weighed portions (0.06 g or 0.2 g) of finely minced animal or human tissue were suspended in 6.0 ml of HS-X6 (10% horse serum in Simm's X6 solution) in a 50 ml Erlenmeyer flask fitted with a rubber stopper. Five hundred units of penicillin and 500 μg of streptomycin per ml were included in the medium of all cultures. SFV was added in 0.24 ml amounts of a $10^{-5.0}$ dilution of mouse brain extract. Cultures were incubated at 37°C for 2 days. The LD_{50} of SFV added to the cultures was determined at the time of each experiment by titrating the extract intracerebrally in mice. After incubation the tissue fragments were extracted in the liquid medium and titrated intracerebrally in mice.

Results. Multiplication of SFV in tissue cultures. SFV multiplied rapidly in tissue cultures containing minced brain or muscle from 8 to 16-day-old chick embryos. The results of a typical experiment employing 0.06 g of 9-day-old chick embryo brain are shown in Fig. 1. Maximum titers ($10^{-4.5}$ to $10^{-6.5}$) were attained within 24 to 48 hours after inoculation and the virus persisted for at least 8 days. The results of experiments designed to determine the capacity of SFV to multiply in tissue cultures of several animal tumors are summarized in Table I. Definite multiplication of virus in 48 hours occurred regularly in 6 to 9-day-old fibroma tissue when 0.2 g of tissue was employed. Growth of virus was not observed in 0.06 g of fibroma tissue. Systematic studies to determine the critical amount of tissue required were not performed. Further experiments revealed that the rate of multiplication of virus in the fibroma tissue was similar to that obtained with chick embryo brain shown in Fig. 1. No multiplication of SFV occurred in cultures of normal adult (6-month-old) domestic rabbit tissues, including brain, heart, kidney, testes, eye, voluntary muscle and subcutaneous tissue. In some instances, however, normal kidney, skin, testicle and subcutaneous tissue from young rabbits (16, 30 and 90 days old) supported the growth of SFV.

The ability of SFV to grow in tissue from several human tumors was also studied. As indicated in Table I, SFV multiplied only in a rhabdomyosarcoma. Of the nonmalignant human tumors studied, SFV grew in 1 of 2 fibromyomas of the uterus but in neither of 2 breast adenofibromas. In a survey of normal human tissue from adults 30 to 73 years old, SFV was also observed to multiply to LD_{50} titers of $10^{-3.0}$ to $10^{-5.3}$ in stomach muscle, hyperplastic thyroid, 1 of 3 endometrial tissues, and in 1 of 4 trials with voluntary muscle. Little or no growth was observed in uterine myometrium, stomach mu-

TABLE I. Multiplication of SFV in Tissue Culture of Certain Tumors.

No. of tests	Tumor	Yield of virus,* Log LD_{50}
13	Rabbit fibroma	4.2–6.6
7	” V_2 carcinoma	<1
16	Mouse sarcoma 180	<1–4.5
4	Hamster fibrosarcoma	<2
1	Human rhabdomyosarcoma	5.3
1	” fibromyosarcoma	<1
1	” lymphoma	<1
2	” breast adenocarcinoma	<1
1	” brain glioma	<2

* After 48 hr incubation at 37°C. Inoculum—log LD_{50} 1-2.6 of SFV.

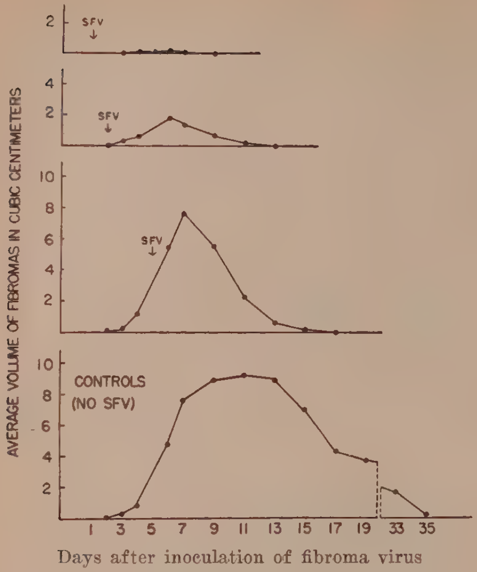

FIG. 2. Development and course of fibromas in control and SFV inoculated rabbits. Each point represents the avg volume of 12 to 16 fibroma growths.

cosa, skin, prostate, fat or tonsil.

Effect of SFV on course of fibromas. The effects of intramuscular injection of SFV on the development and course of rabbit fibromas were studied by inoculating groups of 3 to 4 rabbits with SFV at various intervals after intracutaneous injection of fibroma virus. The results of a comprehensive experiment are shown graphically in Fig. 2. When SFV was injected intramuscularly immediately after the intracutaneous inoculation of fibroma virus, no fibromas developed in about one-third of the inoculated areas. In the remaining areas small papules (2 to 3 mm in diameter) appeared which promptly regressed within 5 days. When SFV was given 24, 48, and 72 hours after fibroma virus inoculation progressively larger fibromas occurred and then disappeared 9, 11, and 13 days respectively after inoculation of fibroma virus. Injection of SFV 5 days after fibroma virus inoculation, when developing fibromas were present, generally caused a firm black scar to develop over the entire fibroma surface within 3 to 5 days. Furthermore, the tumors completely regressed within 13 to 17 days as compared to the average duration of 30 days (range 19-35 days) in the control rabbits. Microscopic examination of sections from the fibromas revealed areas of necrosis 2 days after inoculation of SFV. Within 4 to 7 days all but the deepest layers of fibroma tissue were destroyed. Later injection of SFV up to the 9th day caused similar necrosis of the fibroma surface and somewhat earlier tumor regression but the effect was difficult to demonstrate because of the natural regression of control fibromas. In other experiments it was found that as little as 1.0 cc of a $10^{-6.0}$ dilution of SFV injected 1 day after fibroma virus inoculation caused significant limitation of fibroma growth and regression within 14 days. Intramuscular injection of heat inactivated SFV and normal mouse brain extracts had no effect on the development and course of fibromas. The large amounts of SFV injected into the fibroma rabbits of these experiments was not observed to cause illness or death. The effect of SFV was also demonstrated in hanging drop preparations of fibroma tissue. Pieces of fibroma tissue about 1 mm in the greatest diameter were explanted in plasma hanging drop cultures. Each piece of tissue was embedded in a medium of equal parts of heparinized chicken plasma and 10% chick embryo extract. Six cultures were prepared in a Petri dish(7). Two days after inoculation of fibroma rabbits with SFV, 6 to 9-day fibroma tissue placed in hanging drop preparations showed no fibroblastic proliferations in contrast to moderate fibroblastic proliferation that were usually demonstrated with normal fibroma tissue of comparable age. Also, fibroma tissue fragments that had been incubated 30 to 60 minutes at room temperature in various dilutions of SFV ($10^{-1.0}$ to $10^{-9.0}$) failed to develop fibroblastic proliferation in the hanging drop preparations after exposure to concentrations of SFV from $10^{-1.0}$ to $10^{-5.0}$. This effect of SFV was specifically inhibited by SFV rabbit antiserum.

Recovery of virus from fibroma tissue. Fibromas were removed at intervals after the intramuscular injection of SFV and titrated

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7. Enders, J. F., and Florman, A. L., PROC. SOC. EXP. BIOL. AND MED., 1942, v49, 153.

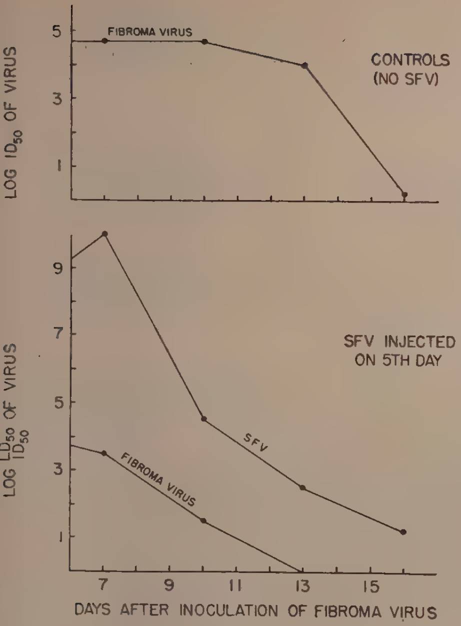

Fig. 3. Recovery of virus from fibromas.

for SFV and fibroma virus. Fig. 3 illustrates the findings in a typical experiment. In this and 2 other experiments SFV persisted until the fibromas completely regressed. The concentration of fibroma virus recovered seemed to parallel the rate of fibroma cell destruction. When necrosis of the fibroma tissue was delayed in the SFV inoculated rabbits, fibroma virus persisted as long as in control animals. This indicated that SFV multiplication *per se* was not associated with a decrease in fibroma virus.

In vitro it was also demonstrated that multiplication and survival of SFV for 8 days in cultures of fibroma tissue did not interfere with the recovery of fibroma virus in concentrations comparable to those obtained from control flasks containing only fibroma tissue. Furthermore, when SFV and fibroma virus were simultaneously added to cultures of voluntary muscle from normal rabbits multiplication of SFV occurred and fibroma virus could be recovered over a 3-day period. This finding is of interest since little or no multiplication of SFV was demonstrated in cultures containing only rabbit voluntary muscle. The results of this experiment suggest that fibroma virus formed fibroblasts or in some way modified muscle cells so that multiplication of SFV was supported.

In titrations for fibroma virus from fibroma tissue that also contained SFV, it was necessary to incubate the dilutions with SFV rabbit antiserum before titration. If this step was omitted SFV, by preventing fibroma growth, masked the presence of fibroma virus.

Discussion. The results of the present experiments demonstrate that Semliki Forest virus multiplies in the rabbit fibroma and destroys the tumor, although no multiplication could be demonstrated in normal subcutaneous tissue. It seems apparent that fibroma virus induces cellular changes in the subcutaneous tissue that are necessary for the growth of Semliki Forest virus. No evidence was obtained in these experiments that interference between the two viruses occurred. Fibroma virus could be obtained from the tumors despite the growth of SFV therein. Microscopic examination of the fibromas and studies of hanging drop preparations revealed that SFV probably exerted its effect by causing necrosis of the parasitized fibroma cells.

The rapid multiplication of SFV to high titer in cultures containing minced tissue provided a relatively simple method for studying the capacity of various tissues to support multiplication of the virus. The finding that SFV multiplied in fibroma tissue *in vitro* led to the present study demonstrating a destructive action of the virus on the tumor *in vivo*. Further studies are in progress to determine the effect of SFV on other tumors, particularly the rabbit myxoma which is so closely related to the rabbit fibroma.

Summary. Semliki Forest virus multiplied in tissue cultures of certain normal and tumor tissues with maximum titers of $10^{4.5}$ to $10^{6.5}$ LD_{50} within 24 to 48 hours. SFV multiplied regularly to high titer in cultures of minced fibroma. Following intramuscular injection of the virus into rabbits bearing virus-induced fibromas, SFV caused rapid necrosis of the tumors. The effect was most apparent when SFV was inoculated simultaneously or within 2 days of the inoculation of fibroma virus.

SFV did not multiply in cultures of normal adult rabbit tissue and caused no deaths or apparent illnesses in rabbits bearing fibromas.

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Factors Interfering with the Hemolytic Property of Mumps Virus.*† (18748)

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In earlier experiments(1), it was observed that amniotic fluids from embryonated eggs containing mumps virus exerted a more powerful and constant hemolytic activity than did virus-containing allantoic fluids from the same eggs(1-3). Allantoic fluids from uninfected eggs were shown to inhibit the hemolytic action of mumps virus and this inhibitory property was not destroyed by incubation with fresh mumps virus for 3 hours at 37°C(3). However, when infected allantoic fluids were dialyzed with phosphate-buffered saline in the cold, their hemolytic activity increased markedly to levels comparable to those obtained with amniotic fluids of similar virus content(3). Furthermore, in the course of earlier experiments(1), it had been observed that an isotonic sodium citrate buffer used as a diluent with infected allantoic fluids resulted in more active hemolysis than when an isotonic phosphate-buffered saline of similar pH was employed. These observations suggested that an inhibitory constituent for the mumps virus hemolysin was present in infected allantoic fluids and that this component was removed by dialysis or sodium citrate. Further studies on the properties and possible nature of this inhibitory factor are reported here.

Materials and methods. An egg-adapted strain of mumps virus was employed for the inoculation of 6- to 8-day-old embryonated eggs by the amniotic or allantoic routes(1). Following incubation at 35°C for 5 to 6 days, the extra-embryonic fluids were harvested separately, placed in glass ampoules, sealed and stored at —70°C. Where dialysis was used, these fluids were placed in cellophane tubing in a large volume of isotonic phosphate-buffered saline (pH 7.2) at 4°C for 24 hours. Hemagglutination and hemolysis tests with chicken erythrocytes were carried out as previously described(2).

Effect of dialysis and diluent. Dialysis of mumps virus-infected amniotic or allantoic fluids with phosphate-buffered saline at 4°C for 24 hours resulted in an increase in hemolytic activity, which was more marked with the allantoic fluids (Table I). Furthermore, the paradoxical increase in hemolysis observed in the first few serial dilutions of untreated allantoic fluids disappeared. An isotonic sodium citrate buffer used as diluent resulted in an increased hemolytic activity with undialyzed allantoic fluid, but had no effect on the dialyzed preparation (Table II). Neither of these procedures had any significant effect on the hemagglutinating activity of the virus.

Effect of an ion exchange resin. The effects observed following dialysis or use of the sodium citrate buffer suggested that some ionic constituent of the allantoic fluid might be re-

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† This investigation was aided by a grant from the Division of Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

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TABLE I. Effect of Dialysis on Hemagglutinative and Hemolytic Activity of Egg Fluids Infected with Mumps Virus.

			Dilution of fluid											Buffer control
Test fluid		Test	2	4	8	16	32	64	128	256	512	1024	2048	
Amniotic	Original	HA*	+	+	+	+	+	+	+	+	+	±	0	0
		HL†	30	27	26	22	10	4	3	0	0	0	0	0
	Dialyzed	HA	+	+	+	+	+	+	+	+	+	±	0	0
		HL	67	57	46	34	16	8	5	4	0	0	0	0
Allantoic	Original	HA	+	+	+	+	+	+	+	+	+	+	±	0
		HL	11	19	20	19	15	4	0	0	0	0	0	0
	Dialyzed	HA	+	+	+	+	+	+	+	+	+	+	±	0
		HL	56	42	30	23	15	8	0	0	0	0	0	0

* Hemagglutination. † Hemolysis expressed as %.

TABLE II. Effect of Phosphate and Citrate Buffers on Hemagglutinative and Hemolytic Activities of Allantoic Fluid Infected with Mumps Virus.

			Dilution of fluid											Buffer control
Test fluid	Test	Buffer	4	8	16	32	64	128	256	512	1024	2048	4096	
Allantoic fluid #1	HL*	Phosphate	7	8	10	1	0	0	0	0	0	0	0	0
		Citrate	37	29	24	13	9	8	4	0	0	0	0	0
	HA†	Phosphate	+	+	+	+	+	+	+	+	+	+	±	0
		Citrate	+	+	+	+	+	+	+	+	+	+	±	0
Allantoic fluid #1 dialyzed	HL	Phosphate	39	28	21	15	9	6	1	0	0	0	0	0
		Citrate	38	30	24	16	7	6	0	0	0	0	0	0
	HA	Phosphate	+	+	+	+	+	+	+	+	+	+	±	0
		Citrate	+	+	+	+	+	+	+	+	+	+	±	0

* Hemolysis expressed as %. † Hemagglutination.

TABLE III. Comparison of Effect of Dialysis and Treatment with Ion Exchange Resin on Hemagglutinative and Hemolytic Activity of Mumps Virus.

			Dilution of fluid											Buffer control
Test fluid	Treatment	Test	2	4	8	16	32	64	128	256	512	1024	2048	
Allantoic	Control	HA*	+	+	+	+	+	+	+	+	+	0	0	0
		HL†	6	5	9	7	4	3	0	0	0	0	0	0
	Dialysis	HA	+	+	+	+	+	+	+	+	+	+	0	0
		HL	46	35	24	18	13	8	6	0	0	0	0	0
	Ion exchange resin	HA	+	+	+	+	+	+	+	+	±	0	0	0
		HL	32	26	22	15	12	5	4	0	0	0	0	0

* Hemagglutination. † Hemolysis expressed as %.

sponsible for the inhibition of the mumps virus hemolysin. Infected allantoic fluid was allowed to flow slowly through a column of a phosphate-buffer washed ion exchange resin (Amberlite I R-100[§]), which was known to remove a number of cations. Comparative effects of dialysis and treatment with this ion exchange resin are presented in Table III. These data indicate that treatment with an ion exchange resin, known to remove such cations as calcium, magnesium and manganese, will eliminate an inhibitor for the mumps virus hemolysin from infected allantoic fluids. Treatment with the ion exchange resin may also result in the loss of small quantities of virus.

Effects of various metallic ions. Previous experiments with dialysis and sodium citrate buffers, as well as a report by Burnet(4) on hemolysis by Newcastle Disease virus, suggested that calcium ions might be responsible for the inhibitory action observed with allantoic fluid virus preparations. Results obtained with the ion exchange resin indicated that calcium, magnesium or manganese ions might

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[§] Obtained from Rohm and Haas Co., Philadelphia.

4. Burnet, F. M., and Lind, P. E., *Aust. J. Exp. Biol. and Med.*, 1950, v38, 129.

TABLE IV. Effect of Calcium and Magnesium and Manganese Ions on the Hemolytic and Hemagglutinative Activity of Mumps Virus

Test fluid	Ion added to PO_4 buffer, mg %	Test	Dilution of fluid												Buffer control
			2	4	8	16	32	64	128	256	512	1024	2048	4096	
Allantoic	0	HL*	23	26	26	24	17	8	5	0	0	0	0	0	0
	Ca-1	,,	11	10	6	5	0	0	0	0	0	0	0	0	0
	Mg-1	,,	23	32	29	24	17	12	7	0	0	0	0	0	0
	Mn-3	,,	21	26	23	16	10	7	4	0	0	0	0	0	0
Allantoic dialyzed	0	HA†	+	+	+	+	+	+	+	+	+	+	+	±	0
		HL	39	25	21	15	9	8	1	0	0	0	0	0	0
	Ca-5	HA	+	+	+	+	+	+	+	+	+	+	+	±	0
		HL	6	0	0	0	0	0	0	0	0	0	0	0	0
	Mg-5	HA	+	+	+	+	+	+	+	+	+	+	+	±	0
		HL	25	20	12	10	6	3	0	0	0	0	0	0	0

* Hemolysis expressed as %. † Hemagglutination.

TABLE V. Effect on Hemolytic Activity of Mumps Virus of the Addition of Calcium to Virus-Infected Allantoic Fluids.

Test fluid	Ca conc added to fluid, mg %	Hemolysin test,* dilutions of fluids									Buffer control
		2	4	8	16	32	64	128	256	512	
Allantoic dialyzed	0	41	33	23	17	10	7	5	3	0	0
	.5	31	30	23	15	12	7	3	0	0	0
	1	24	20	17	17	11	6	3	0	0	0
	2.5	15	16	13	13	9	7	3	0	0	0
	5	14	14	20	18	13	11	4	0	0	0
	10	10	9	10	7	6	0	0	0	0	0

* Hemolysis expressed as %.

be responsible. To determine the relative effects of these various ions, salts of each metal were added to the phosphate-buffer diluent in a series of tests in the concentrations indicated in Tables IV and V.

Calcium in concentrations of 1 to 10 mg % exerted an increasing inhibitory action on mumps virus hemolysis, while manganese at a level of 3 mg % appeared to exert no inhibition and 5 mg % of magnesium had some inhibitory effect. Concentrations of 5 mg % of calcium and magnesium had no influence on the hemagglutinating capacity of mumps virus.

Discussion. Removal of the inhibitory factor for the mumps virus hemolysin from allantoic fluid by dialysis suggests a small particle size, and neutralization or removal by use of sodium citrate buffer suggests calcium as the active agent. The action of the ion exchange resin gives further evidence for the possible role of calcium or other cations. The experiments on the inhibitory effect of various cations indicates that calcium has a potent inhibitory effect on the mumps virus hemolysin. It may be that calcium is the inhibitory substance present naturally in allantoic fluids, but its specific identity with this substance must be further established.

Conclusions. An inhibitor for the mumps virus hemolysin occurring in allantoic fluids of embryonated eggs infected with mumps virus can be removed by dialysis, treatment with a cation exchange resin or use of a sodium citrate buffer as diluent. These results suggest that it is a cation, probably calcium, magnesium or manganese. Of these 3, only calcium has demonstrated any significant inhibitory action on the mumps virus hemolysin.

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Persistence of Virus in Urine as Factor in Spread of Infectious Hepatitis in Dogs.* (18749)

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Although infectious hepatitis virus is not airborne(1), the disease is highly infectious and spreads easily. Susceptible dogs placed with infected ones readily became infected, but when separated by only 6 inches no infection occurred. Further study revealed that ingestion of saliva from infected dogs during the febrile period caused disease. Dogs apparently recovered from the disease still infected those placed in contact with them, although tests then of their saliva, blood and feces revealed no virus. Studies were, therefore, made on other body secretions to determine the source of this persistent virus. Attention focused on the urine and the finding was made that animals eliminated infective virus in the urine long after apparent recovery from the disease. As has been reported for a number of other viruses, its presence in urine might be expected during viremia, but the exceptionally long persistence in the presence of circulating neutralizing antibodies appears an unusual finding for a virus. Although this method of spread is used by leptospira(2,3), it suggests a new concept of virus dissemination.

Methods. Litters of puppies, approximately 1 to 4 months old, were placed in an isolation room so that they would not be exposed to any unplanned infection. At the time of inoculation, the dogs were distributed to individual kennel units separated by a distance of more than 6 inches. Since all dogs in a litter respond in a similar manner to an inoculation of the virus of infectious hepatitis, at least one animal in each litter was shown to be susceptible before others were used for test purposes. Dogs were each inoculated intravenously into the radial vein with 1 cc of a 10% suspension in saline of spleen from an infected dog. After inoculation, temperatures were taken twice each day and a leukocyte count was made daily. Blood was removed from the jugular vein before inoculation and afterwards at daily intervals for 21 days. During this same period, urine was collected daily and thereafter at weekly intervals. All specimens were frozen immediately with a mixture of dry ice and alcohol and placed under dry ice refrigeration. In a test for virus, a specimen was thawed and 1 cc inoculated intravenously into a test dog. Presence of virus was determined by fever, leukopenia and other general signs of illness, finding gross changes in the liver and edema of the gall bladder on autopsy and intranuclear inclusions in histopathological studies. In some instances, dogs were held and given a spleen suspension that had been shown to produce infection in a control animal. Failure to show signs of illness indicated that the test animal had been immunized by virus in the specimen.

Results. As can be seen in Table I, virus was present in 5 tests of blood and in 4 tests of urine made on inoculated dogs during the period of clinical illness. As shown by tests on 5 separate dogs, virus disappeared from the blood at the end of fever and leucopenia but was present in the urine of dogs No. 61 when tested 10 days after inoculation; No. 6, 20 days afterwards; No. 22, 21 days; No. 40, 7, 18 and 41 days; and No. 15, 65, 76, 80, 137 and 161 days afterwards.

Inasmuch as virus was found in urine after recovery, pathological studies were made on the kidneys. Kidneys of puppies examined during the febrile stage showed slight degenerative changes, particularly in the tubules,

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* Aided by a grant from Gaines Dog Research Center.

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TABLE I. Presence and Persistence of Virus in Blood and Urine of Dogs Inoculated Intravenously with Virus of Infectious Hepatitis.

	Clinical signs*				Test for virus		
	Fever		Leucopenia				
Dog No.	Onset	End	Onset	End	Days after inoculation	Blood	Urine
6†	2	6	2	7	20	—	+
15	2	7	3	7	65	N.T.	+
					76	N.T.	+
					80	N.T.	+
					137	N.T.	+
					161	—	+
22†	2	8	3	8	21	—	+
40	2	6	3	6	2	+	—
					3	+	+
					4	+	+
					6	+	+
					7	—	+
					18	—	+
					41	N.T.	+
55	0	0	2	3	3	+	+
61	2	5	2	4	10	—	+

* Indicates days after inoculation.
† Serum contained neutralizing antibodies. N.T. = not tested.

with some protein casts. Inclusion bodies were found in the glomerular endothelium (Fig. 1). In kidneys of puppies that recovered from the disease, widespread but irregularly shaped areas of round cell infiltration were seen, usually in the region of the corticomedullary junction. Adjacent to the patches of cellular infiltration, mononuclear cells were

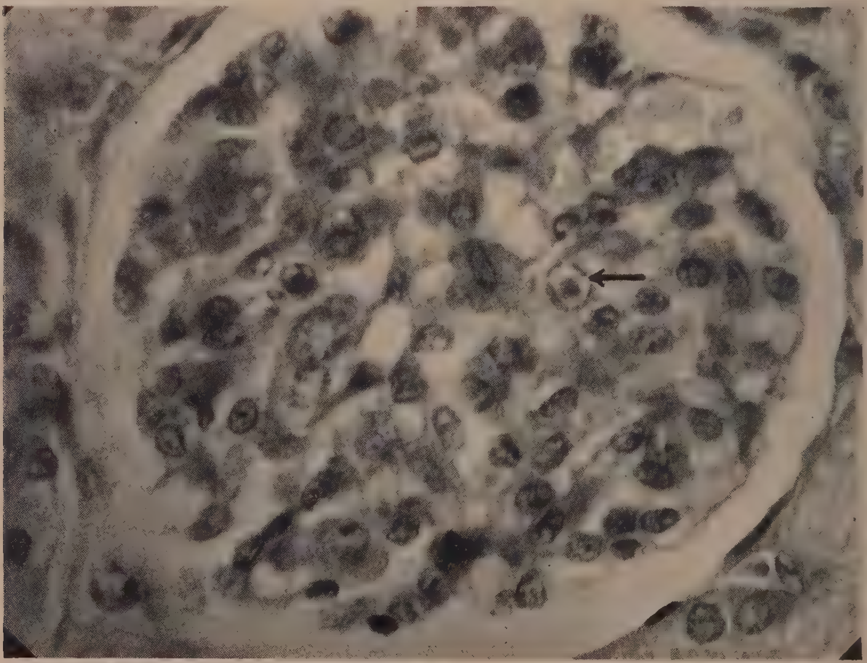

FIG. 1. Kidney from dog showing signs of illness. Note intranuclear inclusion bodies characteristic of the virus of infectious hepatitis.

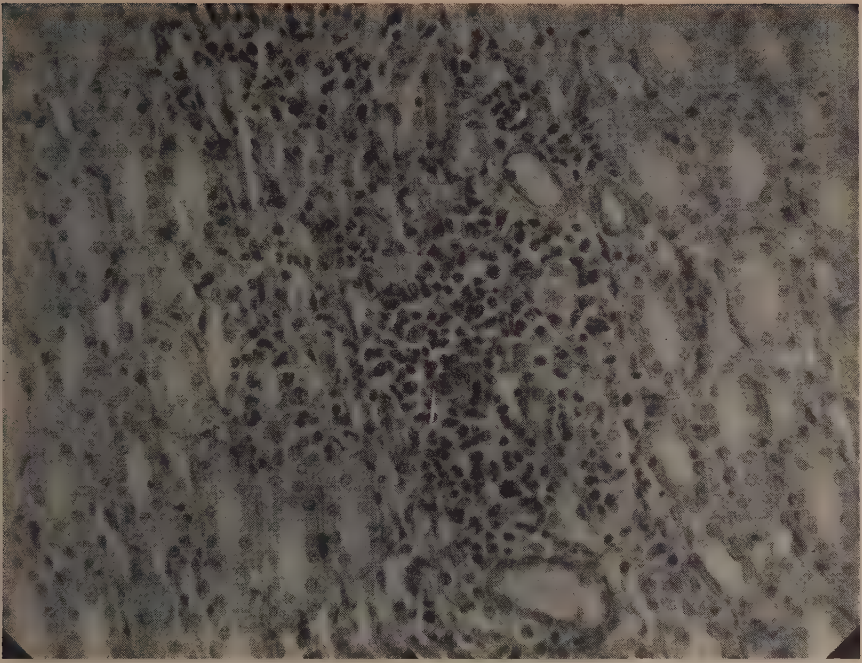

FIG. 2. Kidney from dog after recovery from infectious hepatitis. Note cellular infiltration.

seen between tubules normally occupied by peritubular capillaries (Fig. 2). No evidence of collagen formation or fibrosis was found.

Conclusions. The virus of infectious hepatitis of dogs has been recovered from the urine at intervals of time extending from 3 days after inoculation to at least 161 days afterwards. Inasmuch as blood, saliva, nasal washings and feces from apparently recovered dogs did not contain virus, it appears that infected urine is important in the spread of the disease. Finding a focal interstitial nephritis indicated that the source of virus eliminated in the urine is the kidney.

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Cutaneous Sarcoma-like Lesions of the Horse Caused by the Agent of Bovine Papilloma.* (18750)

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While studying the transmissibility of a cutaneous papilloma of the horse(1) it was found that the horse would respond to the agent of bovine papilloma. The response involved connective tissue rather than epithelial tissue and a sarcoma-like tumor was produced in the skin of the horse. This unusual result constitutes the basis for this report. Papillomas or warts of the skin and oral mucosa occur in a number of different animals(2). Transmission of the disease from animal to animal occurs in nature and virus-like agents have been demonstrated in experimental animals(3). These agents have been regarded as active only in the same species of animals from which the material was obtained. While a number of the common laboratory animals have been exposed to infective material from different types of papillomas, we find no record in the available literature of exposure of the horse to bovine papilloma material.

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The bovine papilloma material was obtained by biopsy from active spontaneous cases in cattle. The material came from 4 different outbreaks of the disease. In each instance the material was tested on susceptible calves and found capable of producing typical bovine papillomas. The inoculums were prepared by grinding papilloma tissue with saline solution (approximate ratio: 1 to 15). The supernatant fluid was used for inoculation after the coarser particles of tissue had settled. Both fresh and glycerinated material was used. Eleven horses (6 mares, 2 geldings, and 3 stallions) varying in age from 1 to 3 years were exposed. Inoculations, in duplicate, were made intradermally with about .01 ml of the prepared inoculum. Exposures were also made on scarified skin of 5 of the 11 horses.

Results. A small firm fibrous nodule could be detected at the point of intradermal inoculation in 12 to 27 days. These nodules increased in size at variable rates (Fig. 1 and 2). The nodules attained their maximum size (2 to 3 cm in diameter) in 30 to 50 days. The hair covering tended to disappear as these grew larger and sometimes the surface was devoid of epithelium, leaving a raw area. The nodules disappeared 100 to 150 days after their appearance in most horses. The tumor lasted for more than 600 days in one horse (Fig. 1, horse 12). The other extreme was a horse in which the tumor lasted only 25 days and attained a maximum size of 1 cm in diameter (Fig. 1, horse 1). Only one horse (a 2-year-old female) failed to respond to the inoculation.

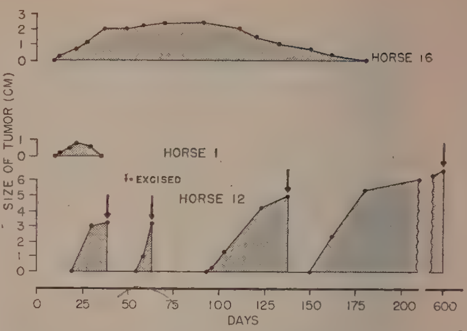

FIG. 1. Diagrammatic representation of rate of growth of sarcomas induced in skin of experimental horses. Each dot represents a point of observation. The record for horse 16 is typical of the results obtained. The record for horse 1 indicates a very small short-lived tumor in the skin of this animal, which was an unusual result. The record for horse 12 indicates unusually large size of the sarcoma induced in this animal and recurrence of growth following excision of the mass at 3 different times.

Attempts to inoculate onto scarified skin were generally negative. Small wart-like growths of brief duration occurred in only one of 5 horses thus exposed. Bacteria-free filtrate through a Mandler filter at about 10-lb pressure produced sarcoma-like tumors in one horse. These were smaller and of shorter duration than the tumors produced with unfiltered bovine wart material. Both fresh and glycerinated bovine wart material were active. Some glycerinated material that had been stored for 2 years was still active.

The sarcoma-like tumor induced in horse 12 grew rapidly. The 2 sites of exposure were close to each other and soon coalesced into one tumor mass (Fig. 3). The entire tumor was excised 3 times and rapidly recurred, each time growing a little larger than formerly (Fig. 1). A fourth attempt at surgical removal was made about 600 days after inoculation. This was again by simple excision. Examination of the removed tumor indicated

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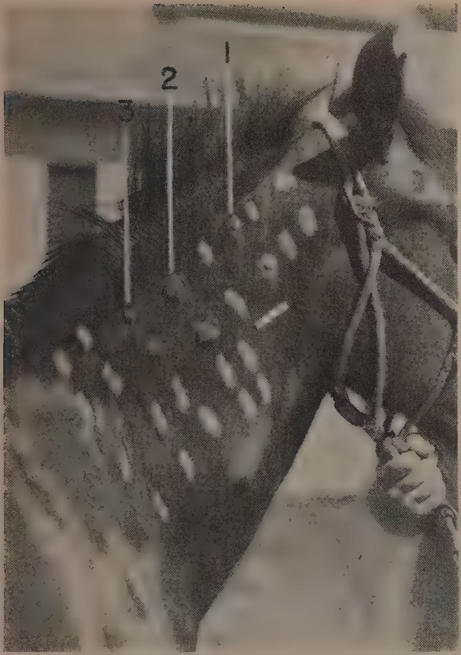

Fig. 2. Photograph of the sites of inoculation on skin of horse 16, 72 days after inoculation, at the points indicated by 1: the pair (vertical) of inoculations with filtrate from bovine papilloma material, 2: the pair of inoculations with ground bovine papilloma material, and 3: the pair of inoculations with experimentally produced material from horse 12. The other shaved areas were sites of inoculations with other materials.

that some active growth was present at the edges of the mass. Within a week there was some evidence of recurrence of the growth and debridement of the entire wound area was done with an electro-cautery. There was no recurrence following the last operation. Material from this growth was used to inoculate other horses and a similar tumor was reproduced. Generally, these nodules were somewhat smaller than the lesions induced with bovine wart material. The tendency to recur following surgical removal was noted in four other experimental horses. There was no evidence of acquired immunity since the horses have been susceptible to repeated exposure.

The sarcoma-like nodules were extremely firm and made up of irregular interlacing bundles of connective tissue fibers (Fig. 4). The appearance of the connective tissue cells varied somewhat from nodule to nodule and also in different parts of the same tumor. In some areas they were stellate and rather loose. In other areas the nuclei were smaller, fewer in number, with relatively more collagenous cytoplasm in the cells. Mitotic figures were quite numerous in the more rapidly growing parts of the tumor and the tissue had a definite anaplastic appearance. In some sections there was an irregular infiltration with polyblasts and polymorphonuclear leukocytes due to a superimposed secondary bacterial infection. Growth of the tumor probably began where the inoculum had been placed. Growth was confined to the corium. It extended upward to the basement membrane of the epithelium and sometimes caused its destruction. There was no evidence of spread of the growth either as internal metastases or to other points of the skin of the experimental animals.

The pathological similarity between the experimental condition and the condition re-

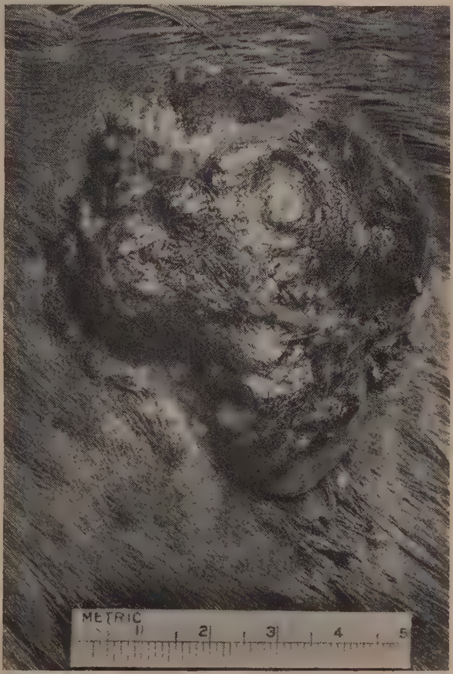

Fig. 3. The sarcoma induced on the skin of the neck of horse 12 with bovine papilloma material. This was the growth which recurred and was present 6½ months after inoculation and 50 days following last of three surgical removals of the mass.

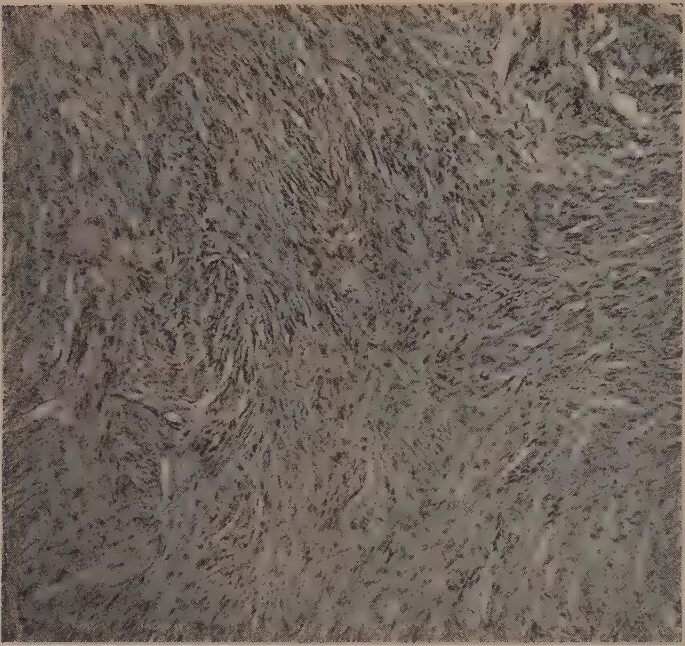

FIG. 4. Photomicrograph of tumor removed from horse 12, 600 days after inoculation with bovine papilloma material. The fibroblastic character of the growth with considerable collagenous cytoplasm is illustrated. Magnification ×120.

ported as equine sarcoid(4,5) and "Schwann tumor"(6) is striking. Glycerinated material from one field case of equine sarcoid was placed on 4 "susceptible" horses. Growth like that from bovine wart material developed in only one horse at the 2 sites of intradermal inoculation. This is additional evidence of similarity of the two conditions.

Summary. Material derived from spontaneous field cases of bovine papilloma of the skin was capable of causing a sarcoma-like lesion in the skin of the horse. The tumors so induced have some characteristics resembling equine sarcoid. The ability of an oncogenic agent to induce an epithelial tumor in one animal and to cause a new growth of connective tissue cells in another species is unique.

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Serologic Cross-Reactivity of Some Enterobacteriaceae Isolated in the United States with Cholera Vibrios. (18751)

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The identity of a microorganism is usually determined by morphologic, physical, biochemical and serologic studies. Especially in the practical diagnosis of cholera vibrios fast serologic identification is much desirable, due to the character of the disease. Colonies

TABLE I. Cholera Agglutination Tests with Non-Cholera Organisms Selected at Random in the Chicago Area.

Organism	No. tested	No. positive in spot tests with serum against Inaba	Ogawa
E. coli	70	11	2
A. aerogenes	134	26	1
Paracolobactrum	5	1	0
Proteus	29	2	0
Pseudomonas	55	2	0
Alcaligenes	6	0	0
Streptococcus	16	7	2
Total	315	49	5

grown on initial isolating plates which resemble those of vibrios, may directly be tested by slide agglutination. Those grown on media containing carbohydrates and indicators, however, may cause difficulties, since they frequently show dissociation. Serologic cross-reactions with some strains of Salmonella and Alcaligenes may be expected, especially when sera with high titers are employed(1).

In order to ascertain whether non-cholera organisms frequently present in stools give such reactions also in countries outside of the cholera area, 315 microbes were picked out at random from patients with diarrhea in Chicago and used for slide agglutination tests with Inaba and Ogawa "O-H" sera prepared in this laboratory according to the routine method(2). Table I shows the results. As many as 49 (*i.e.*, 15.5%) were agglutinated by the Inaba serum. Five of these organisms reacted also with the Ogawa serum. Agglutination experiments employing the serial dilution test-tube method and high titer sera gave positive reactions with seven (*i.e.*, 2.2%) of the examined organisms.

Further experiments were carried out by using sera prepared by injecting rabbits with formolized and heat-killed *V. comma* cultures, respectively. These more specific sera had titers of 1:1,280 and 1:2,560 against their homologous antigens. Heat-killed antigens prepared from one *E. coli* (No. 95) and one *P. morgani* (No. 18) strain were agglutinated by dilutions 1:40 to 1:160 of the serum prepared by injecting heat-killed Inaba vibrios, while formolized antigens prepared from 5 strains of *A. aerogenes* (Nos. 3, 5, 19, 20 and 23), as well as from *P. morgani* (No. 18) were agglutinated by dilutions 1:40 to 1:160 or 1:320 of the serum prepared by treating rabbits with formolized Inaba cultures. While the proportion of strains of Enterobacteriaceae reacting to significant titers with cholera sera is low, the occurrence of such cross-reactions has to be kept in mind in diagnostic work.

Since formalin does not destroy the "O" antigen of cholera vibrios, these results must be evaluated rather carefully. It seems, however, that a factor which is absent in pure "O" cholera antigens and also in Ogawa sera produced by the injection of formolized vibrios into rabbits was present to a significant level in 5 *Aerobacter aerogenes* cultures and one *Proteus morgani* culture. This seems to be the common antigen "A" of Inaba and Ogawa strains the existence of which was recently confirmed by Kauffmann(3).

Further studies were carried out to ascertain the serologic relationship of these Enterobacteriaceae to Salmonellae and Brucellae, which are known to harbor antigenic fractions common with *V. comma*(1,4,5).

Cholera antigens were prepared according to the procedure of Ranta and Dolman(6). In order to obtain pure "H" sera, the "O-H" sera were absorbed with "O" antigens. Brucella and *S. enteritidis* sera were prepared according to standard methods(2).

Table II shows the results of agglutination tests which confirm former observations and indicate possible common antigenic factors appearing in cholera vibrios, Brucellae, Salmonellae, and some Enterobacteriaceae.

Reciprocal absorption experiments were carried out. The results of part of them are

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TABLE II. Cross-Agglutination Tests with Cholera and Non-Cholera Serums and Antigens.

	Agglutination titer with serum						
Antigen	*Br. suis*	*Br. melitensis*	*S. enteritidis* ''O''	*S. enteritidis* ''H''	Inaba ''O-H''	Inaba ''O''	Inaba ''H''
Br. suis	2560	5120	0	80	320	40	120
Br. melitensis	2560	5120	0	80	320	40	120
S. enteritidis ''O''	0	0	2560	160	640	120	0
S. enteritidis ''H''	40	80	0	10240	320	0	80
Inaba ''O-H''	640	640	320	640	5120	5120	640
Inaba ''O''	0	0	320	640	2560	2560	0
E. coli #95	0	0	40	0	160	160	0
A. aerogenes #3	40	80	0	40	320	0	80
P. morgani #18	40	80	80	40	160	160	160

TABLE III. Absorption Experiments with Inaba ''O-H'' Serum.

	Agglutinin titers after absorption with						
Antigen used for agglutination	None	*Br. suis*	*Br. melitensis*	*S. enteritidis*	*E. coli* # 95	*A. aerogenes* #3	*P. morgani* #18
Inaba ''O-H''	5120	2560	2560	1280	2560	2560	1280
Inaba ''O''	2560	2560	2560	1280	1280	1280	1280
Br. suis ''H''	320	0	0	0	320	160	160
Br. melitensis ''H''	320	0	0	0	320	80	160
S. enteritidis ''O''	640	640	640	40	80	640	80
E. coli #95	160	40	0	40	0	160	0
S. enteritidis ''H''	320	0	0	40	320	80	160
A. aerogenes #3	320	40	40	40	0	0	80
Pr. morgani #18	160	40	0	40	0	160	0

shown in Table III. They proved a fraction of the cholera "H" antigen to be identical with the factor common to the "g,m" antigen of Salmonellae and a part of the Brucella flagellar antigen, while a common "O" antigenic component was found to be part of the I.IX.XII antigen of Salmonellae.

After absorption with *S. enteritidis* (I.IX. XII:g,m. . .) the Inaba and Ogawa "O-H" sera did not agglutinate Brucellae which were agglutinated before absorption to a titer of 1:320. Absorption of these sera with *Br. suis* and *Br. melitensis* eliminated the agglutination of formolized but not of heat-killed *S. enteritidis* previously observed in serum dilutions to 1:80. Inaba "H" serum absorbed with the *A. aerogenes* cultures which were agglutinated by it (see Table I) did not agglutinate *Br. suis, Br. melitensis* or *S. enteritidis*. Neither did the Inaba "O" serum agglutinate heat-killed *S. enteritidis* after absorption with the *E. coli* No. 95 or *P. morgani* No. 18 strains. It was concluded, therefore, that some antigenic factors are common to *V. comma,* Brucellae, Salmonellae, and the examined Enterobacteriaceae.

Thus the list of organisms agglutinated by cholera serums has to be extended to include also other Enterobacteriaceae than Salmonellae.

Summary. Of 315 microorganisms selected at random from stool cultures of persons in Chicago, 49 or 15.5% showed cross-reactions with diagnostic cholera sera when examined with the slide test, and 7 or 2.2% with the serial test-tube dilution method. The common antigen seems to be the "A" fraction of *V. comma* and part of its "H" antigen, which also occurs in Brucellae and Salmonellae.

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In vitro Sensitivity of Recently Isolated Cholera Vibrios to Ten Antibiotics. (18752)

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The activity of antibiotics against *Vibrio comma* is usually determined in American and European institutions using relatively old stock strains. *V. comma* cultures maintained in laboratories, however, frequently undergo changes. It was observed during preliminary experiments(1,2) that the susceptibility of these organisms to antibiotics increases during storage. It was decided, therefore, to examine 53 cholera vibrio strains which were received shortly after isolation in Egypt, India and French Indochina, for the estimation of the sensitivity of *V. comma* to antibiotics.

The tube-dilution method was employed in these experiments. Duplicate series, containing decreasing amounts of the respective antibiotics, were set up and inoculated with measured amounts of an 18- to 20-hours-old broth culture. All tests were carried out using Penassay Broth, Difco, and read after 20 to 22 hours incubation at 37°C.

Penicillin G, chloromycetin, neomycin, terramycin, polymyxin D, bacitracin and prodigiosin were received from the Commercial Solvents Corporation, Terre Haute, Indiana. Penicillin O (Cer-O-Cillin) and streptomycin were supplied by the Upjohn Co., Kalamazoo, Michigan. Aureomycin was furnished by the Lederle Laboratories, Pearl River, N. Y.

The results of these experiments revealed considerable variations in the susceptibility of cholera vibrios to the different antibiotics according to the geographical origin of the strains. Table I, which summarizes the outcome of these tests, shows, therefore, the results with regard to the place wherefrom the vibrios were received.

Cholera vibrios from the Bombay area were susceptible to all antibiotics tested in this series. Ogawa types were, on the whole, somewhat more resistant to antibiotics than Inaba strains. Inaba type organisms from Bombay were less sensitive to neomycin, bacitracin and aureomycin, but more susceptible to terramycin and polymyxin D than strains from other areas. While resistant organisms were not received from Bombay or Egypt, several strains from Bengal and Assam, as well as

TABLE I. *In Vitro* Susceptibility of 53 Cholera Vibrios to Antibiotics According to Geographical Origin.

Strains	Inaba	Inaba	Ogawa	Inaba	Ogawa	Inaba	Ogawa
No. tested	3	5	5	10	10	10	10
From	Egypt	Bombay	Bombay	Bengal	Bengal	Indochina	Indochina
Antibiotic	Mean inhibitory dose in μg or units in ml medium						
Penicillin G*	25	20	22.5	35.5†	50.5‡	54.2‡	60.1‡
” O	25	14.5	16.25	30.6†	42.8‡	31.5†	55.7‡
Streptomycin	10.3	25	14.5	28.3†	29.1†	22.5†	30.2†
Chloromycetin	5.3	1.57	3.2	.9†	4.1†	3.7†	3.9†
Aureomycin	.23	2.82	3.4	.7†	4.9†	.4†	.8†
Neomycin*	.36	4.07	4.37	.62	.72	1.62	1.75
Terramycin	2.5	2	3	2.81	3.18	—	—
Polymyxin D*	12.26	1.62	.96	4.32†	6.62†	—	—
Bacitracin	1.5	100 ‡	100 ‡	2.91	4.88	18.5†	22.4†
Prodigiosin	.5	.6	1.12	—	—	—	—

* In units.
— Not tested.
† One to 2 strains resistant to 100 units or μg.
‡ Three to 5 strains resistant to 100 units or μg.

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from French Indochina, were not sensitive to most antibiotics in doses smaller than 100 units or μg per ml test fluid. Organisms resistant to neomycin or terramycin were not encountered. The number of strains tested with prodigiosin is small but the results are encouraging.

Summary. Fifty-three recently isolated strains of *V. comma* were tested against 10 antibiotics. The results showed a wide variation of susceptibility to these drugs, according to the geographical origin of the vibrios.

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Some Early Nuclear Effects of Ionizing Radiation.* (18753)

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The cellular changes which follow ionizing radiation have been most extensively investigated in relation to mitoses. It is noted that within 12 minutes after exposure to heavy doses, mitotic inhibition may occur, and the chromosomes involved show clumping, fragmentation and lagging(1). Nuclear chromatin degenerates and giant nuclei may result. These relatively temporary effects are seen particularly in the mitotic prophase, and the different susceptibilities of various cell types is well established. Cytoplasmic alterations such as vacuolization and massing of the Golgi network and fragmentation are observed, but major interest still attaches to nuclear changes. Various mechanisms have been invoked in explanation. The idea that ionizing radiation prevents formation of desoxyribose nucleic acid from ribose nucleic acid, thus inhibiting chromosome synthesis(2), has also some support from investigation of chemical agents which inhibit prophase onset and are hence termed "radiomimetic." According to Dustin(3) these agents produce sequentially (a) cytoplasmic swelling, (b) alteration of nuclear membrane, (c) accumulation of thymonucleic acid on nuclear membrane with fragmentation and disappearance of all chromosomes, (d) spherical massing of basic protein and ribonucleoprotein, with persisting nucleolus, then (e) rapid fragmentation with extrusion of debris from the cell.

Present studies. As a preliminary to histochemical study of this problem, a series of rats injected intraperitoneally with large doses of P^{32}† were killed at hourly intervals. Lethal radiation effects consisting of nuclear pyknosis and fragmentation were usually observed in lymphoid tissues, intestinal crypts, bone marrow and gonads. In contrast to and at times preceding this lethal type of radiation reaction, nuclear swelling was observed in many tissues. In those sensitive cells mentioned above it proceeded to cell death, whereas in other organs such as liver, kidney and perhaps brain, nuclear swelling disappeared within 24 hours. Thus the more radioresistant tissues appeared to show a nonlethal reversible radiation effect.

With the conventional formalin- or Zenker-fixed paraffin embedded tissues, however, nuclear detail is not adequately preserved to permit of an accurate study and interpretation of such relatively slight nuclear alterations. The simplified freezing-drying technic devised in this laboratory for autoradiography(4), and since used with success in histochemical problems(5), has proved useful in this situation. Feulgen stains of normal and irradiated tis-

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* This work was done under U. S. Atomic Energy Commission Contract AT (30-1)-901 with the New England Deaconess Hospital.

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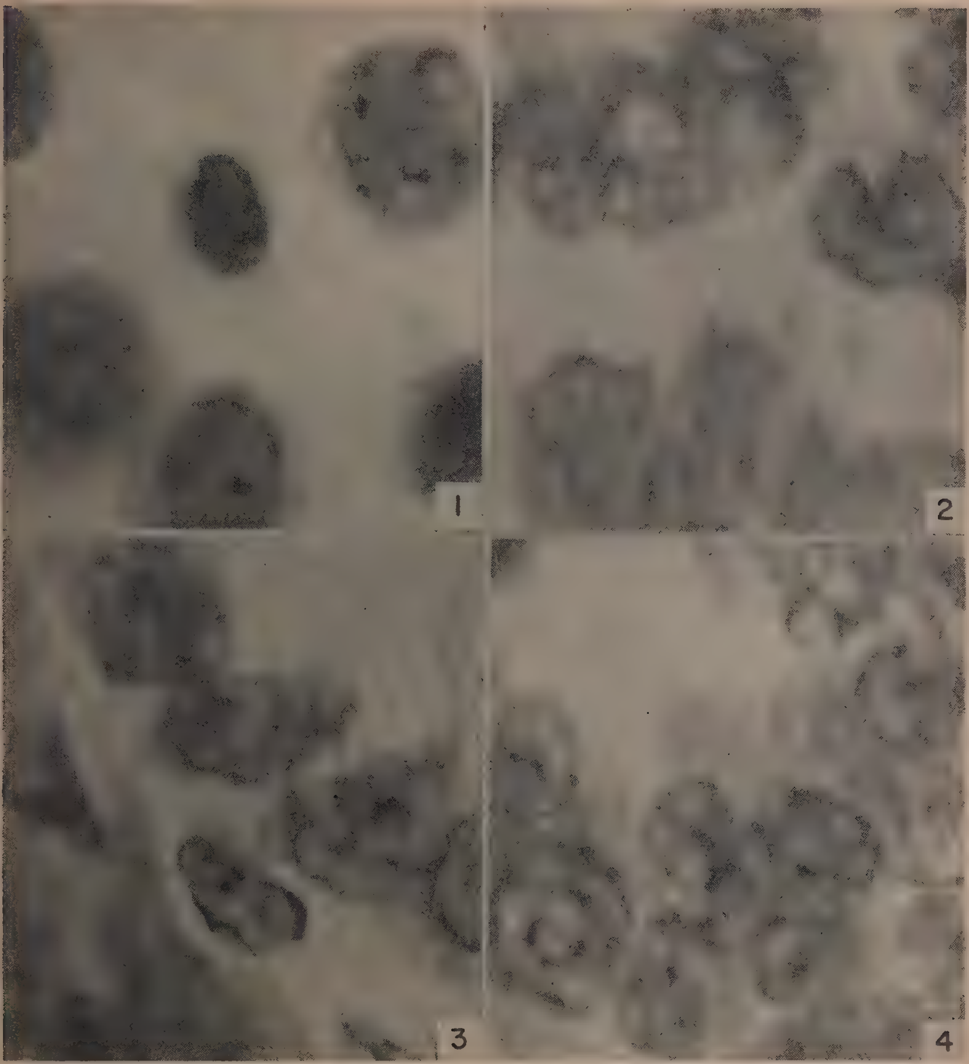

Frozen-dried, dry-mounted tissues, photographed in polarized light to reduce halation. Feulgen stains, ×3000.

Fig. 1. Nuclear details of normal mouse liver cells, showing vacuoles. Dark chromatin masses present are often eccentrically vacuolated on microscopic examination.

Fig. 2. Untreated mouse sarcoma #180. Nuclear vacuoles are numerous and prominent in sarcoma cells, producing the lacy pattern shown.

Fig. 3 and 4. Epithelial nuclei of small intestinal crypts, 3½ hr after subcutaneous injection of 249 μc of P^{32} into a normal 65 g rat. Large irregular intranuclear vacuoles are observed, irregularly stretching nuclear membranes. Cytoplasm is abnormally coarse and thready.

sues were examined from groups of rats and mice, some of which were exposed to 200 KV X-radiation; or the 90-120 MEV neutron beam, with an ionizing component reduced below the LD_{50} by means of lead shields, from the Harvard cyclotron; or P^{32} injection. Slides were prepared by the dry mounting

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method which prevents loss of water soluble substances. Such preparations have excellent cytologic detail surpassing that of ordinary histologic technic and comparable to the appearance of living cells in phase-contrast microscopy. The advantages of specific staining and relative permanence can be achieved.

Nuclei of normal mouse liver (Fig. 1) kidney, and untreated mouse sarcoma 180, among other tissues, contain numerous vacuoles covered with Feulgen-positive material, and the nucleoli are often eccentrically vacuolated. Nuclear membranes are seen as sharp smooth lines. Nuclear vacuoles of neoplastic cells are larger and relatively numerous (Fig. 2).

Soon after radiation, for example 3½ hours after 249 μc of carrier-free P^{32} injected subcutaneously into rats, striking nuclear changes occur, which are more conspicuous in radiosensitive cells. Epithelial nuclei of intestinal crypts develop giant clear vacuoles surrounded by irregular layers of Feulgen-positive material. The nuclear membranes are irregularly thinned and stretched by the clear vacuoles within, which apparently explode from the nuclei with lethal effect (Fig. 3,4). The nucleolar vacuolization is likewise abnormally exaggerated. Relatively radiosensitive neoplasms such as mouse sarcoma 180 acquire similar intranuclear vacuoles after irradiation, as for example by neutron beam of cyclotron origin. The size of the vacuoles produced exceeds those of any normal tissue examined. Both in contrast to the appearance of conventional histologic preparations of radiated tumor and to unirradiated tumor prepared by freezing-drying every nucleus shows vacuolation (Fig. 5,6).

Less extreme but equally significant intranuclear vacuolization occurs within 24 hours in radioresistant cells such as those of liver. Enlarged clear vacuoles distend the nucleus or push against the blurred nuclear membranes and are apparently extruded. The nuclear membranes appear rough, stain unevenly, and become fibrillar and poorly defined in places. A majority if not all liver cells are affected (Fig. 7, 8). About 24 hours after injection of P^{32}, the non-lethal nuclear radiation effects tend to subside in liver, but with persistence of an occasional nucleus with vacuoles and relatively less content of Feulgen-positive material.

Changes observed by others(2,6), and by ourselves both in conventional and frozen-dried tissue preparations, include a moderate general increase in nuclear volumes not involving vacuolization, decreased Feulgen staining of nuclear chromatin, sap and membranes, and cytoplasmic swelling. Chromosomes in mitoses stain abnormally densely, and appear clumped and unusually "sticky" in later phases of cell division.

Later radiation effects have not as yet been investigated with this technic.

Discussion. The major investigations into radiation effects upon cells have been concerned with lethal alterations of sensitive cells, such as those of lymphoid, hematopoietic and gonadal tissues. To understand the acute radiation syndrome(7) with its signs of general toxemia, it may be well to extend our studies of the less marked nonlethal changes observed in physiologically important tissues such as the liver. The newer freezing-drying and histochemical technics now available make possible the demonstration of relatively slight morphologic changes, which, as in the liver, may accompany important alterations in metabolic activity.

While processes of equal importance to the nuclear synthesis of nucleic acid compounds may remain undiscovered, the present study confirms the belief that radiation adversely affects the ribose and desoxyribose nucleic acid components which are Feulgen-positive. At this time, it seems logical to ascribe some radiation effects on mitoses to interference with production and metabolism of such Feulgen-positive nuclear constituents.

Summary. Frozen-dried, dry mounted Feulgen stained preparations of irradiated rat and mouse tissues were employed to study early cytologic changes after ionizing radiation. Fine nuclear details are preserved by the freezing-drying method. Lethal radiation effects observed in radiosensitive cells include

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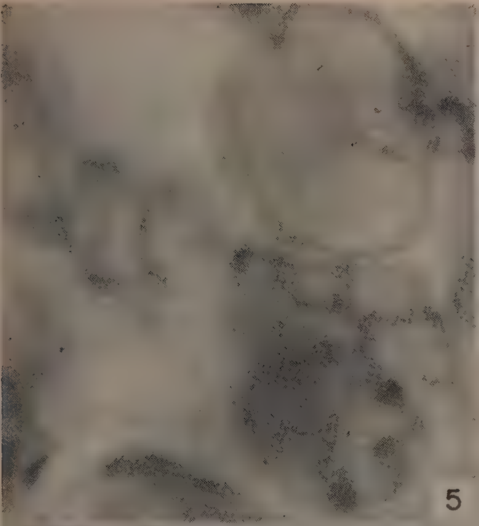
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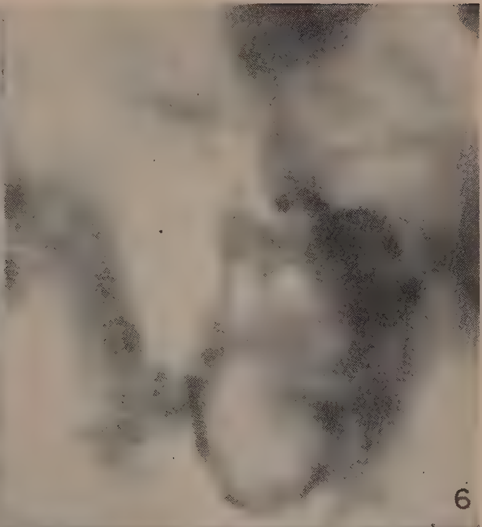
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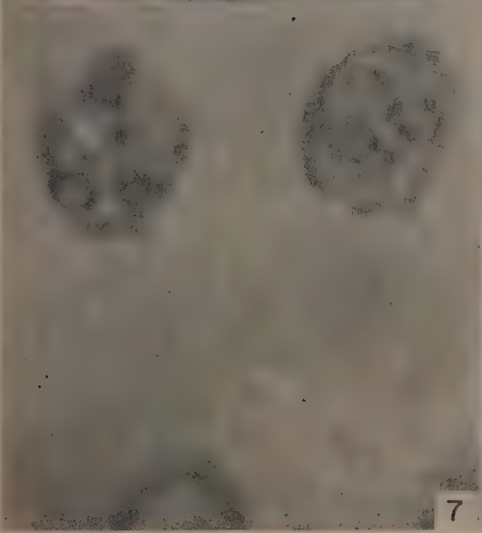
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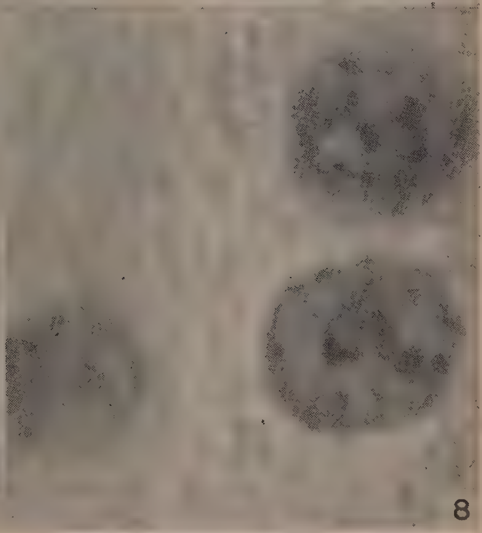
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FIG. 5 and 6. Mouse sarcoma #180, 24 hr after sublethal exposure to cyclotron beam consisting primarily of neutrons of the 90 to 120 MEV range. Giant intranuclear vacuoles are uniformly present, with extreme nuclear swelling. Some nuclei are composed almost wholly of one or two vacuoles.

FIG. 7 and 8. Liver cells, 3½ hr after subcutaneous injection of 249 μc of P^{32} into a normal 65 g rat. Nuclei contain sharply outlined vacuoles, some touching nuclear membranes. Cytoplasm appears abnormally coarse and vacuolated.

formation of large intranuclear vacuoles with stretching of nuclear membranes. Nonlethal changes in radioresistant tissues comprise smaller intranuclear vacuoles, decreased Feulgen stain of nuclear chromatin and sap, and loss of regular sharp staining of nuclear membranes. Such changes in liver cells may contribute to the acute radiation syndrome. Histochemical technics may provide a better understanding of tissue changes after ionizing radiation.

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Post-Radiation Parabiosis and Survival in Rats.* (18754)

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A variety of measures employed before or during whole body exposure to X-ray has been shown to afford partial protection against the lethal effects of irradiation. These procedures include administration of estradiol 10 days prior to irradiation(1,2), administration of sulfhydryl compounds immediately before exposure(3,4), pre-irradiation parabiosis(5), shielding of the spleen or other organs(6) and anoxia(7) during irradiation. Measures employed after irradiation have been less successful. Antibiotics(8) exchange transfusion(9) and DOCA(10) have been reported to reduce mortality, but no published data are available on the efficacy of these 3 therapeutic measures after doses of radiation which would be uniformly fatal in untreated animals. The present experiments were undertaken to determine whether a post-irradiation procedure can be of benefit in animals exposed to a uniformly fatal dose of whole body irradiation.

Methods. Radiation was delivered by a Picker therapy X-ray machine operating at 200 KVP (25 ma. 1.0 mm Al, 0.25 mm Cu, TSD 50 cm, field size 20 x 20 cm, HVL 0.8 Cu, dose rate 44 r in air per minute). Half of each total dose was delivered to the dorsal and half to the ventral surface of the rats in order to make irradiation more uniform throughout the body.

Female Sprague-Dawley rats weighing 80-120 g were used in all experiments. Parabiosis was accomplished under nembutal anesthesia. Silk was used in the first experiment and monofilament nylon No. 0 in the 2 subsequent experiments to hold the scapulae and femurs of the parabions together. The skin was joined with skin clips.

Results. In the first experiment, 8 female parabions joined 6 weeks before irradiation, were used. One partner was irradiated with 700 r while the other was shielded with 4 mm of lead. By measurement, the shielded rat received less than 1% of the incident radiation. Three pairs died on the 5th, 14th, and 33rd days respectively after irradiation. The 5 surviving pairs were separated 34 days after irradiation and are alive 5½ months later. At the time of irradiation the partners were essentially the same weight. At the present time the irradiated members weigh less, but in other respects appear normal. Fertility has not been tested. The 10 single control animals for this experiment all died from the effects of the 700 r dose, 9 of them by the 10th day and the 10th on the 19th day.

In the second experiment 14 rats were irradiated with 700 r and joined to non-irradiated litter mates within a few hours after exposure to X-ray. The parabions and single irradiated controls were treated with 15,000 units of penicillin, 20 mg streptomycin, and 2 cc intraperitoneal saline daily for the first 3 days after irradiation in an effort to avoid deaths from dehydration associated with early diarrhea, from wound infection, and from shock. Eight of the 14 pairs died at 1, 2, 4, 6, 8, 9, 11, and 26 days after irradiation. The surviving 6 pairs were separated on the 28th day. Of these, 1 irradiated animal died on the

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* The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the view of the Navy Department or the Naval Service at large.

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45th day after irradiation. The remaining 5 are alive and gaining weight 3½ months later. The irradiated animals weigh less than their litter mates, but appear otherwise normal. The 10 single controls had all died by the 14th day.

In the third experiment, designed to provide tissues for histologic examination, 12 irradiated animals were joined to non-irradiated litter mates within a few hours after irradiation. Twenty-four litter mates of these pairs served as controls. Antibiotics and saline were given to all animals as in the preceding experiment. Nine of the 12 parabiotic pairs and 11 of the 24 single controls were alive on the 10th day after irradiation. Five parabions and 5 single animals were killed on the 10th day, and the remaining 4 parabiotic and 6 single rats on the 11th day. At autopsy the mesenteric and cervical lymph nodes of all single rats were hemorrhagic. No hemorrhages were noted in any of the irradiated parabions. On histologic examination, the femoral bone marrows of the single animals were largely atrophic and showed only scattered centers of myelopoiesis of varying size. In contrast, regeneration of the bone marrow in irradiated parabiotics was usually diffuse, and the overall cellularity often approached that of normal femoral marrow. In 2 of the parabiotic animals the cellularity of the bone marrow was within the range of normal.

In addition to the 20 animals used as controls in the first 2 experiments, 84 female rats of comparable age and weight were exposed to 700 r. Thirty of these rats were treated for 3 days with saline, penicillin, and streptomycin as in our 2nd and 3rd experiments. None of these animals survived beyond the 22nd day. Statistical analysis showed that there is only one chance in 20 that the true mortality of a 700 r dose for these rats is less than 97% and only 1 chance in 100 that it is below 95%. An additional 45 animals were exposed to doses of radiation from 325-625 r. The dose-mortality data are presented in Table I. It may be concluded that 700 r is either uniformly lethal for this age group of the Sprague-Dawley strain or approaches the uniformly lethal dose very closely.

TABLE I. Dose-Mortality Data for Rats Exposed to 325-700 r Whole Body X-radiation.

Dose in r	No. of rats exposed	No. dead at 30 days
325	9	0
400	9	2
475	9	4
550	9	8
625	9	8
700	104	104

Mortality in probits = −1.0508 + .01266 r. S. E. of slope = .00198. Estimated LD_{50} = 478 r ± 18 r. Estimated LD_{99} = 662 r ± 29 r.

Discussion. The above experiments confirm earlier findings that parabiosis performed some time before irradiation results in survival of the unprotected parabion after exposure to an otherwise fatal dose of X-ray(5). This protective effect of pre-irradiation parabiosis might be considered as similar to the shielding of part of an individual animal. However, as demonstrated here, parabiosis performed after irradiation is still of benefit in spite of the additional stresses of anesthesia and trauma. Post-radiation parabiosis must therefore be considered as akin to a therapeutic procedure.

Death following the doses of ionizing radiation used in these experiments is primarily though not solely, due to the sequelae of pancytopenia. It appears reasonable to assume that the survival of the parabiotic rats is linked to the accelerated regeneration of their bone marrow. This accelerated regeneration of hemopoiesis may be due to one or more of 3 mechanisms: transfer of some material necessary for the orderly differentiation of primitive mesenchymal cells, transplantation of blood-forming cells from the non-irradiated partner, or "detoxification" of a hypothetical substance produced by radiation in the exposed parabion. Since a "toxin" has never been satisfactorily demonstrated as playing a major role in radiation injury, the last possibility appears remote.

Summary and conclusions. Rats can survive an otherwise fatal dose of radiation if joined after radiation to non-irradiated litter mates. Such rats show accelerated bone marrow regeneration when compared with single controls. It is suggested that post-radiation parabiosis is primarily of benefit because it accelerates recovery of myelopoiesis by the transfer of some cellular or non-cellular material from the non-irradiated partner.

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Assay of Plasma Antihemophilic Activity in Normal, Heterozygous (Hemophilia) and Prothrombinopenic Dogs.* (18755)

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The corrective action of normal plasma on the delayed clotting of hemophilic blood is attributed to its content of antihemophilic factor (AHF). It has been shown(1-3) that the amount of prothrombin consumed in whole hemophilic blood is directly proportional to the amount of AHF added. On the basis of this relationship, an assay procedure for AHF (2,3) was recently developed. In this paper, a series of studies on AHF are reported in which an improved assay procedure was used. The new method is more sensitive than the previous one, and it permits expression of results in per cent of normal control plasma. Studies have been made on the AHF level in normal dogs and in female dogs heterozygous for hemophilia, as well as in animals with hypoprothrombinemia due to liver injury and to dicumarol administration.

Assay of antihemophilic activity. The plasmas being tested for AHF were prepared from citrated whole blood (9 parts blood and 1 part 3.2% sodium citrate) in an angle centrifuge at about 3000 g for 20 minutes, and then dialyzed ("Visking" cellulose casing) with continuous agitation against 0.032% sodium citrate in 0.9% NaCl solution for 2 hours at 4°C. The normal control and test plasmas were diluted varying amounts (1-2 to 1-50) with saline. To 0.1 ml of each dilution of each plasma were added 0.15 ml imidazole buffer pH 7.25, and 1 ml freshly drawn hemophilic dog blood. A single hemophilic dog, either male or female(4), was used for each assay. After a reaction period of 20 minutes (28°C), 0.15 ml 3.2% sodium citrate was added to each tube, and the serum (or plasma) expressed by immediate centrifugation. Residual prothrombin was determined by the two-stage method(5), with added Factor V(6) prepared from dog plasma. The results were expressed in per cent (citrated plasma from the hemophilic dog = 100%). The prothrombin is plotted against the calculated amount of native test or control plasma added to 1 ml native hemophilic plasma. Fig. 1 illustrates the method used for comparing AHF in the test plasma with

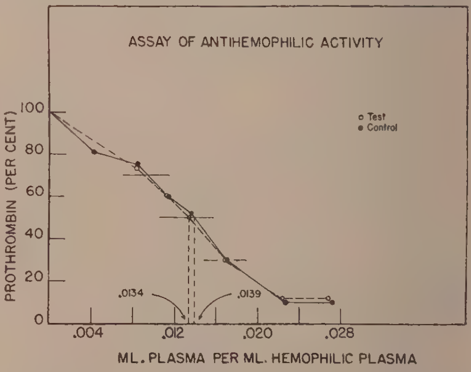

FIG. 1.

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TABLE I. Comparative Plasma AHF Levels in Normal Dogs and Female Dogs Heterozygous for Hemophilia.

Test dog			Control dog		AHF
Type	No.	Sex	No.	Sex	% of control plasma
Normal	1	♀	2	♂	89
	4	♂	3	♂	93
	5	♀	3	♂	101
	5	♀	3	♂	106
	6	♂	3	♂	92
	6	♂	3	♂	103
Heterozygous	8	♀	3	♂	80
	9	♀	7	♀	108
	10	♀	5	♀	87
	10	♀	5	♀	105
	10	♀	5	♀	101

the normal control plasma. The test plasma in this illustration was from a dog 6 days after prolonged chloroform anesthesia (see Fig. 2A). The amount of each plasma required to give a residual prothrombin of 70, 50 and 30% respectively in the hemophilic substrate was estimated by interpolation. For each prothrombin level the value, ml control plasma/ml test plasma × 100 is determined. This value represents the AHF in the test plasma in per cent of that contained in the control. In the example shown, these values were 107, 104 and 101%, giving an average of 104%.

AHF levels in normal dogs and dogs heterozygous for hemophilia. Table I shows the results obtained in 6 experiments with normal dogs. In each experiment a pair of dogs of either sex was tested for plasma AHF. One animal was arbitrarily designated as the test animal, the other as the control. It will be seen that, under these circumstances, the AHF in the normal test animals varied from 89% to 106%. In another set of similar experiments, non-bleeder females, heterozygous for hemophilia (Table I), were compared with normal males or females. Again, it will be noted that the AHF was nearly the same in the two sets of plasma. The heterozygous females varied from 80 to 108% of the normal controls.

Liver injury. Liver damage was produced by chloroform anesthesia in 6 normal dogs. Each animal was fasted for 24 hours and then subjected to deep anesthesia for 90-120 minutes. Fig. 2A shows the results of one experiment. Frequent determinations of plasma prothrombin (2-stage) and plasma AHF were made. No appreciable change occurred in the AHF level of plasma, although the prothrombin fell rapidly and then gradually returned to normal, as is characteristic of acute liver damage(5). In 2 of the 6 experiments, fresh citrated plasma was obtained at the time the hypoprothrombinemia was most severe, and was transfused into hemophilic dogs. The average results of the 2 experiments are given in Fig. 2B. The degree of improvement of the clotting defect in the hemophilic dogs was determined from the prothrombin utilization rates(7,8); the residual prothrombin values of serum one hour after collecting the hemophilic blood are plotted. The corrective effect was immediate and was still evident about 5 days later. It will be noted that the plasma from the dogs with liver injury was about as effective as normal plasma. The correction *in vivo* by plasma from the chloroform-treated dogs, low in prothrombin and without detectable fibrinogen, confirms the findings with the *in vitro* assay procedure.

Dicumarol. Hypoprothrombinemia was induced by dicumarol in 3 normal dogs. The results of one experiment in which assays for plasma AHF were performed frequently are shown in Fig. 2C. No significant change in antihemophilic activity of the plasma occurred, although the prothrombin fell to a low level. In 2 of the experiments, prothrombinopenic plasma from the dicumarol-treated dogs was transfused into hemophilic dogs. This plasma was as effective in correcting the clotting defect of hemophilic animals as was normal plasma (Fig. 2D). Thus it appears that dicumarol administration likewise has no effect on plasma AHF.

Discussion. The assay procedure outlined furnishes a sensitive and accurate method for determination of antihemophilic activity of

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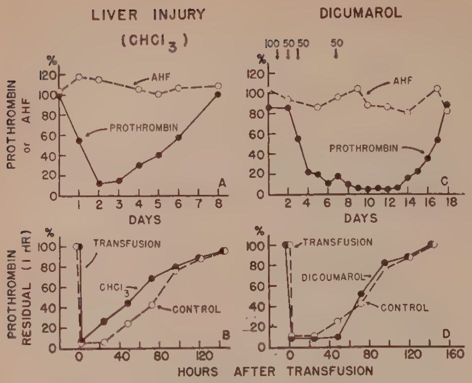

FIG. 2A. CHCl$_3$ anesthesia administered for 105 min. to dog 11 ♂, 19.1 kg, after fasting 24 hr. Normal control plasma (100% prothrombin and AHF values) obtained from Dog 3 ♂, 23.3 kg.

FIG. 2B. Transfusions of hemophilic dogs with plasma from CHCl$_3$-treated and normal dogs. CHCl$_3$ anesthesia for 2 hr and 90 min. respectively to dog 3 ♂, 25.2 kg and dog 12 ♂, 11.3 kg. Citrated plasma from the 2 CHCl$_3$-treated dogs (2 days post-anesthesia, prothrombin $< 5\%$) and from a normal dog (two separate preparations, dog 4 ♂, 22 kg) transfused to hemophilic dogs 13 ♂, 20 kg, 14 ♂, 20 kg, 15 ♂, 21.3 kg and 16 ♂, 20 kg respectively, 2.6 ml plasma per kg body wt. Prothrombin utilization rates of hemophilic whole blood determined before and after transfusion(1).

FIG. 2C. Dicumarol administration to dog 4 ♂, 22 kg. Dosage in mg indicated on chart. Normal control plasma (100% prothrombin and AHF values) obtained from dog 3 ♂, 23.3 kg.

FIG. 2D. Transfusions of hemophilic dogs with plasma from dicumarol-treated and normal dogs, 2.6 ml per kg body wt. One "dicumarol" plasma from dog, Fig. 2C, on 9th day. Other "dicumarol" plasma from dog 1 ♀, 19 kg, given 100 mg dicumarol 1st day, 50 mg 2nd, 3rd, and 4th days. Citrated plasma obtained on 5th day (prothrombin 8%). Hemophilic dogs 15 ♂, 21.3 kg and 17 ♀, 19 kg used as recipients. Normal plasma transfusions as in Fig. 2B.

plasma. However, the procedure is moderately laborious, since as many as 15 to 20 separate two-stage prothrombin tests are required for a single determination of AHF. Although only canine hemophilic blood was used as a substrate in the experiments above, human hemophilic blood has been used by us in similar studies, and it appears to give equally satisfactory results. Under the standard conditions of the AHF assay, an approximately linear relationship was found to exist between the amount of AHF added to hemophilic blood and the amount of prothrombin consumed (Fig. 1). This relationship may provide a basis for the definition of a unit of antihemophilic activity. Tentatively, it is suggested that one unit of AHF be defined as that amount needed to convert 10 units of prothrombin per ml plasma under standard conditions in the assay procedure. Since dog plasma contains about 400 units of prothrombin per ml with the method used, and since about 0.013 ml normal plasma was required to convert 50%, or 200 units, of prothrombin in the assay procedure (Fig. 1), then normal dog plasma would contain roughly 1500 AHF units per ml.

The data in Table I indicate that a relatively steady state of plasma AHF exists in normal dogs, regardless of sex. This is true also for females heterozygous for hemophilia. The normal AHF levels in the heterozygotes appear to rule out the possibility of their identification by merely determining the plasma content of this clotting factor.

The failure of liver damage to reduce the plasma AHF suggests that this factor is not produced in the liver. Likewise, the stability of AHF during dicumarol treatment indicates that it is synthesized by a mechanism much different from that for prothrombin. It is of interest that plasma used for transfusions in the liver injury experiments contained little or no fibrinogen. In one experiment, the blood did not clot, either spontaneously or after addition of thrombin. Yet the plasma AHF was fully active when transfused into a hemophilic dog. This is another example of dissociation of fibrinogen and AHF(3), clotting factors with similar physical properties.

Summary. 1. A method is described for the assay of antihemophilic factor; results may be expressed in per cent of a control plasma or in units. Hemophilic blood, canine or human, is used as substrate. 2. In normal dogs, regardless of sex, and in females heterozygous for hemophilia, a steady state of plasma AHF appears to exist. The antihemophilic activity of heterozygotes is approximately the same as that of normal dogs. 3. The antihemophilic activity of plasma is maintained at normal levels following acute liver injury, as well as after dicumarol administration.

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Hypoprothrombinemia Induced by Gossypol.* (18756)

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Several investigators(1-3) have reported that cottonseed meal or gossypol feeding causes hemorrhages in pigs, rabbits, and rats. In repeated feeding tests in this laboratory hemorrhages occurred in weanling rabbits on rations containing 20% cottonseed meal. Although the hemorrhages were usually evident in the first loop of the small intestine they were sometimes found in the long bones of the legs, in the heart muscle, or around the brain. These observations led to an investigation of the effect of gossypol and cottonseed meal on clotting time, the results of which are reported here.

Methods. Clotting time was determined by a slight modification of the method of Sternberger(4). In this modification thrombinized plasma was diluted to 9.1% of normal plasma. At this dilution and by this procedure the normal clotting time of rabbits and pigs is 8 ± 1 seconds. Gossypol was dissolved in peanut oil for administration by stomach tube to rabbits and pigs except in the direct comparison with Dicumarol when both compounds were dissolved in NaOH and the solutions adjusted to pH 9.4 with HCl; the latter operations were carried out in an atmosphere of N_2. Cottonseed meal containing 0.1% free gossypol was fed at 20% of the ration to weanling rabbits (700-800 g) in 28-day feeding trials. The other components of the basal ration were ground corn or barley 72%, alfalfa meal 7%, and mineral mixture 1%.

Results. The increase in clotting time of rabbits (Fig. 1, Curve A), after administration of gossypol in oil at 3 mg/kg, from 8 to 18 seconds in 16 hours corresponds to a decrease in prothrombin of about 35% as estimated from a dilution curve for normal plasma. Although few pigs are represented, the response under these conditions (Fig. 1, Curve B) was somewhat greater in this species than in rabbits. When compared directly on the same rabbits, gossypol (Fig. 1, Curve C) was somewhat more rapid in action than dicumarol (Fig. 1, Curve D) but the latter induced a greater response of longer duration. The approximately 66% higher molecular weight of gossypol may account for some of the difference between the two substances.

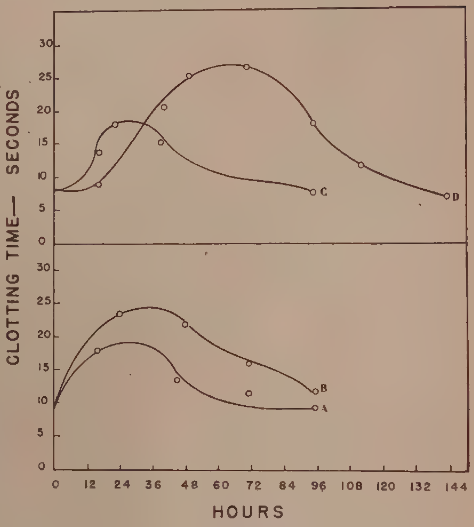

FIG. 1. Effect of gossypol on prothrombin of rabbits and pigs. Administered by stomach tube at 3 mg/kg. Curve A, in peanut oil, avg for 20 rabbits; curve B, in peanut oil, 6 pigs; curve C, in alkaline solution, 6 rabbits; curve D, dicumarol in alkaline solution, 3 mg/kg, same 6 animals as in curve C.

The effect of cottonseed meal feeding on prothrombin of rabbits is illustrated in Fig. 2. This curve is a composite obtained from several 28-day feeding trials with cottonseed meal at the 20% level. In these tests various supplements were added without appreciable effect. The points represent irregular numbers of animals—the first is an average of 96 and the last 58. This drop is due

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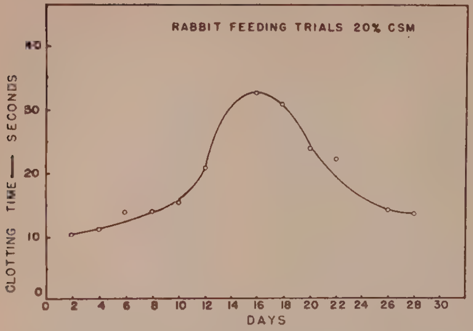

FIG. 2. Effect of cottonseed meal feeding on prothrombin of rabbits. Each point represents avg of preceding and designated day. Avg for over 50 animals at the 2nd, 8th, 12th, 22nd, and 28th days.

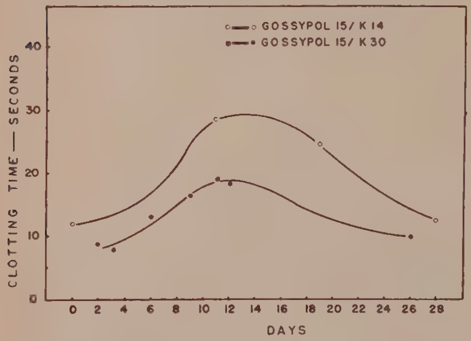

FIG. 3. Effect of vit K supplements on prothrombin of rabbits on 20% cottonseed meal rations. Upper curve; Synkavite added at 14 mg. K/15 mg free gossypol. 8 rabbits. Lower curve; 2-methyl 1,4 naphthoquinone added at 2 mg/1 mg free gossypol. 8 rabbits.

largely to losses, and, after the 14th day this curve more and more shows the response of surviving animals. Most of these rabbits had a calculated intake of about 20 mg of gossypol in the first 2 days; yet the effect on clotting time did not appear until about the 7th day when some of the animals had ingested 70 mg gossypol. This delayed effect is in sharp contrast with that of the direct administration of gossypol at 3 mg/kg. In the feeding tests, of which these were a part, 44 of 103 deaths occurred between the 14th and 21st day when the delay in clotting was around the maximum. However, about 20% of the deaths occurred between the 21st and 28th day of the feeding period and hemorrhages were usually evident even though clotting time approached a normal value. Around the 28th day, the clotting time of 6 animals ranged from 10 to 14 seconds within the 72 hours preceding death. Moreover, 3 animals, which were hemorrhagic, showed clotting time of less than 12 seconds within the 24 hours preceding death which occurred between the 9th and 12th day of the feeding test.

The effect of vit. K supplementation on the hypoprothrombinemia induced in rabbits by the same cottonseed meal is shown in Fig. 3. The upper curve shows that Synkavite, a water soluble form of the vitamin, had no effect on the hypoprothrombinemia induced in rabbits when added to the ration at 14 mg to 15 mg gossypol. The lower curve was obtained when 2-methyl 1,4-naphthoquinone was added to the ration at the rate of 2 mg/1 mg gossypol. Peanut oil was also added to this ration to bring the fat content from about 2.0 to 7.0% Although the added fat might have been a factor there was a decided reduction of the gossypol effect with this form of the vitamin at this very high level. Vitamin K in either of these forms did not prevent hemorrhages or death in these tests.

Discussion. Perhaps the hemorrhagic properties of cottonseed meal have not been emphasized heretofore because hemorrhages are not so apparent in many species or in older animals and their widespread occurrence after cottonseed meal feeding can only be established by histological studies. However, with sensitive young rabbits as the test animal, hemorrhages are more obvious.

The delayed effect of so-called free gossypol in cottonseed meal on clotting time as compared with the relatively rapid action when given directly can hardly be explained on the basis of poor absorption alone. The rather slow increase in clotting time after the inception of the hypoprothrombinemia followed by the slow return toward normal suggests a cyclic response much more complex than might be accounted for by a simple absorption mechanism. The fact that hemorrhages were evident in rabbits which died in the fourth week of the feeding period on 20% cottonseed meal rations when the clotting time approached normal in addition to the reduction of the hypoprothrombinemia by high vitamin

K supplementation without preventing hemorrhages and death, is evidence that hypoprothrombinemia is not the primary cause of hemorrhages and of cottonseed meal toxicity. This affords an interesting parallel to the conclusion of Schofield(5) regarding hemorrhagic sweet clover disease long before Dicumarol was isolated and characterized.

Summary. Gossypol induces hypoprothrombinemia in rabbits and pigs. Cottonseed meal containing free gossypol induces hypoprothrombinemia in young rabbits which begins after about 7 days feeding and passes through a maximum at approximately 17 days then slowly returns to normal around 28 days. Gossypol is not as effective an anticoagulant as Dicumarol but acts somewhat more rapidly.

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Received March 29, 1951. P.S.E.B.M., 1951, v77.

Failure of Aureomycin in Treatment of Experimental Leishmaniasis (18757)

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Aureomycin has recently been demonstrated both experimentally and clinically to be effective against amebiasis(1-4), trichomonas vaginalis vaginitis(5) and malarial infestations(6,7). It is, therefore of interest to know whether this antibiotic will also act on another parasitic infection, namely, Leishmaniasis (*Leishmania donovani*).

Materials and methods. Chinese hamsters, weighing 20 to 30 g each, were infected by intraperitoneal injection of 0.2 ml of a saline suspension of leishmania, prepared by mixing 30 ml of sterile saline solution with a finely ground spleen, freshly removed from a heavily infected hamster. The animals were divided into 2 groups and each group was further divided into 3 subgroups of 20 animals each. Aureomycin* was given intraperitoneally twice a day, *i.e.* 9 a.m. and 5 p.m., for 5 days for group A, for 10 days for group B. For the former, treatment was started immediately after the injection of leishmania, and for the latter, the antibiotic was not given until 2 months after infection. The dosage schedule of aureomycin was carried out according to the following plan:

Subgroup 1. 3 mg per animal per day.
Subgroup 2. 1.5 mg per animal per day
Subgroup 3. 0.5 ml normal saline per animal per day and served as infected, untreated controls.

Results. During the period of treatment, 8 hamsters in group A-1 and 10 in group B-1 died, apparently due to drug intoxication: Post-mortem examination of those animals revealed the presence of hemorrhages in the lungs with extensive peritoneal adhesions. All the rest survived and were killed 2 months after the completion of the treatment. The degree of parasitization was determined by the microscopic examination of the smears made from spleens at autopsy. Results obtained during the present investigation failed

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* The aureomycin used in this study was supplied by Lederle Laboratories Division of American Cyanamid Co., N. Y.

to show any appreciable difference between the treated and untreated animals of both groups. All the surviving animals were shown to be heavily infected with L.D. bodies and their spleens were uniformly found to be enlarged. It may thus be concluded that aureomycin in a dose as high as 100-150 mg per kilo body weight per day failed to exert any beneficial effect upon experimental Leishmaniasis in Chinese hamsters.

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The Effect of Protein Depletion upon the Enzyme Activities of Organs.* (18758)

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The gross attributes of the syndrome of protein depletion in animals are now generally recognized; perhaps less evident are the underlying metabolic changes. One approach to this problem has been a study of the nitrogen exchange of the body during depletion(1,2). The activities of enzyme systems, which ultimately may dictate the major features of depletion, constitute another approach to the deeper mechanisms involved. Therefore this study was designed to screen some of these systems in rats on low-protein and protein-free diets.

Depletion of animals. Young adult albino rats were housed in self-cleaning cages in an air-conditioned room. The control diet "G" was composed of 18% casein, 68% dextrose, 8% hydrogenated vegetable oil, 4% salts (USP No. 1), and 2% cod liver oil. Low-protein diet "E" contained only 2% casein, while diet "D" was protein-free; in both these cases the dextrose content was increased to substitute for the protein omitted. In addition, the rats were fed orally an adequate daily supplement of pure vitamins, which included the "B" components, para-amino benzoic acid, choline, inositol, 2-methyl-1,4-naphthoquinone and biotin.

The rats were maintained on these diets for varying periods, depending upon when their tissues were analyzed for enzyme activity. Although it was originally planned to examine for a gradual change in such activity with time, it was noted on examination of the data that any changes which did occur were of the same order of magnitude after 3 weeks of depletion as they were later on. For this reason, the depletion values have been grouped for analysis and comparison with the simultaneously measured control values. Tests of significance were performed, where possible, by the 't' test(3). In the determination of enzyme activity maximum effort was directed toward making analyses under identical conditions. Thus strict time schedules were observed for autopsy, treatment of tissue and measurements. Especial care was taken with homogenization of the tissues which was always performed in the same manner(4).

Adenosinetriphosphatase (ATP-ase) of skeletal muscle. Analyses were performed according to the method of Dubois and Potter (5); the adenosine triphosphate was prepared according to Kerr(6). In Table I are re-

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† Present address: Ortho Research Foundation, Raritan, N. J.

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TABLE I.

Enzyme system	Measurement	Diet	No. of Rats	Values (Mean ± S.D.)
Adenosine triphosphatase of skeletal muscle	μg phosphate split/mg dry wt	G. Control	6	40.4 ± 10.9
		E. Low-protein	6	40.2 ± 14.3
		D. Protein-free	4	41.9 ± 10.7
Cytochrome oxidase of liver	μl O_2 used/hr/mg dry wt	G. Control	5	73.4 ± 25.9
		E. Low-protein	5	62.5 ± 17.5
		D. Protein-free	4	79.4 ± 14.9
Arginase of liver	Arginase units x 10^{-2}/g	G. Control	6	38.1 ± 6.5
		E. Low-protein	4	36 ± 4.1
		D. Protein-free	6	38.1 ± 6.5
	Arginase units x 10^{-2}/total liver	G. Control	6	256.8 ± 48.7
		E. Low-protein	4	196.9 ± 35.4
		D. Protein-free	5	168.6 ± 37.7
Succinoxidase of liver	μl O_2 used/hr/mg dry wt	G. Control	12	56.2 ± 11.3
		E. Low protein	9	26.8 ± 10.6
		D. Protein-free	10	21.8 ± 13.7

ported the results for animals maintained on the diets from 28 to 58 days. Values are expressed as the micrograms phosphate split from the substrate per milligram dry weight of muscle. There is no apparent difference in ATP-ase activity of muscle with varying levels of protein in the diet.

Cytochrome oxidase of the liver. Cytochrome C was prepared by the method of Keilin and Hartree(7) from horse heart tissue, and standardized spectrophotometrically(8). The p-phenylemediamine method was used to measure activity(9,10). Table I summarizes the results with the three levels of protein feeding, expressed as the microliters of oxygen used per mg weight of tissue per hour. Here again there was no apparent change in activity with diet.

Arginase activity of liver. The liver arginase activity was determined by a modification of the methods of Hunter and Downs(11) and Lightbody(12). Activity was expressed as units according to a standard curve for ammonia liberation and weight of tissue used (13). One unit is taken as the activity yielding 0.34 mg ammonia under the standard conditions of the assay. It is evident from the values in Table I that the arginase activity per gram of liver remains relatively constant, while the decrease in liver weight with depletion is reflected in a reduced total arginase activity. These results may be compared with those of Lightbody and Kleinman(14) who reported some decrease in liver arginase activity on 6% protein diets, and with those of Rosenthal *et al.*(15) who reported as much as a 70% loss in activity after two weeks on protein-deficient diets.

Succinoxidase activity of liver. Measurement of the succinoxidase activity of liver was performed by the method of Schneider and Potter(16). There is a marked decrease in the succinoxidase activity with protein depletion in the rat, as shown in Table I. This effect was already evident after the third week of depletion, when tests were first performed, and may actually occur much earlier than this. The addition of 0.25% methionine

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to the 2% protein diet did not elevate the value in 3 additional animals tested (Mean 26.8 ± 11.7). If the values for each diet are grouped and statistically tested(3), the P values for each depletion group compared to the control group are less than 0.01, indicating significant differences. Potter and Klug (17) have reported that succinate oxidation of liver was decreased on diets high in carbohydrate. Since the high carbohydrate diets contained only 6% casein as protein, there exists the possibility that the observed effect might be due in large part to this low protein diet.

Summary. Rats fed low protein or protein-free diets for periods up to 8 weeks exhibit (1) no change in skeletal muscle ATP-ase activity; (2) no change in liver cytochrome oxidase activity; (3) no change in liver arginase activity per gram of tissue, but a decrease in total arginase units; and (4) a marked decrease in the succinoxidase activity of liver.

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Relation of Protein Nutrition to the Healing of Experimental Wounds.* (18759)

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A number of reports have indicated that the level of protein intake has a marked effect on the rate of healing of wounds(1-8). There is, however, little work relating protein metabolism or the effect of amino acids to the healing of wounds(4-6,9,10). It has been shown that methionine will increase the rate of healing of experimental wounds(4), whereas tryptophane, valine and lysine have no significant effect on the rate of healing(10). Since greater nitrogen retention will occur when extra methionine is fed(11,12), it seems probable that the methionine, rather than other essential amino acids, is the limiting factor in the healing process. In fact, injured dogs will retain methionine sulfur and incorporate it into protein, even when a negative nitrogen balance exists(13). Beyond the fact that a negative nitrogen balance appears shortly after wounding(14,15), little seems to be known about protein metabolism during the healing of wounds.

Further study may help elucidate the role of protein metabolism in the healing process. The present paper attempts to relate nitrogen and sulfur metabolism to the rate of healing of experimental wounds.

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* This work was done under contract with the U. S. Navy, Office of Naval Research.

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TABLE I. Composition of Diets.*

Group #	% casein	% gelatin	% supplemental methionine	g N/100 g diet
I	20	0	0	2.56
II	6	0	0	.76
III	6	11.3	0	2.55
IV	6	11.3	.147	2.57

* Sucrose content of diets was adjusted so that all diets provided same number of calories/100 g diet.

Experimental. In these experiments, four groups of 24 female albino rats were used. The initial average weight of the animals in each group ranged from 170 to 186 g. The animals in Group I were fed the following basal diet: sucrose, 63 g; casein, 20 g; lard, 10 g; corn oil, 2 g; salt mixture,[†] 5 g; thiamine HCl, 1 mg; riboflavin, 1 mg; pyridoxine HCl, 1 mg; calcium pantothenate, 4 mg; nicotinic acid amide, 15 mg; inositol, 5 mg; 2-methyl naphthoquinone, 0.5 mg; choline chloride, 25 mg; vitamin A,[‡] 1500 IU; vitamin D,[‡] 210 IU. The other groups of animals were fed diets, modified from the basal, as indicated in Table I. All the diets were isocaloric and contained the same amount of vitamin supplement as in the basal diet. Each animal was presented with 15 g of diet per day. The residual uneaten food was weighed to determine the daily food consumption. All animals were given distilled water, *ad libitum*. The animals were first accustomed to the synthetic diets for 4 days. Then they were wounded under nembutal and ether anesthesia. After clipping the hair over the area of the shoulder blades, a skin incision, 2 cm in length, was made with a sharp scalpel in the mid-dorsal region. The edges of the wound were opposed and held in place with Michel wound-clips (11 mm), which were left in place during the experiment. At approximately weekly intervals, 8 rats from each group were killed and the tensile strength of a 0.5 cm section of the healing wound was determined. The method of measuring the tensile strength of such wounds has been previously described(1).

The rats were weighed and 24-hour urine samples were collected regularly, during the course of the experiment. The urine samples were stored under toluene at 5°C until the analyses were made. The urine was analyzed for total nitrogen (microkjeldahl), urea nitrogen(16), amino acid nitrogen(17), and total sulfur(18).

Results and discussion. Previous papers have indicated that a negative nitrogen balance appears directly after animals have been subjected to trauma(9,13-15). A sharp decrease in the amount of nitrogen retained, shortly after the animals were wounded, was observed in these experiments, although not all the animals went into negative nitrogen balance. The sulfur balance remained positive and essentially constant throughout the experiment, regardless of the changes in the nitrogen balance. By the tenth day after wounding, the daily nitrogen retention had returned to approximately the level observed before the wound was made. This is illustrated in Table II.

A marked decrease in urinary urea output (from 109 mg per day to 81 mg per day) with a concomitant increase in the amino acid nitrogen output (from 6.1 mg per day to 26.6 mg per day) was observed shortly after the

TABLE II. Nitrogen and Sulfur Retention Before and After Wounding.

Group #	Avg retention before wounding*		Retention on 2nd day after wounding*		Retention on 10th day after wounding†	
	N, mg	S, mg	N, mg	S, mg	N, mg	S, mg
I	92	11.3	44	11.2	85	10.1
II	9	2.8	–11	1.5	8	2.3
III	34	6	14	3.8	39	6.6
IV	52	9.2	15	11.1	42	13.4

* Computed from analyses obtained from 24 animals per group.

† Computed from analyses obtained from 16 animals per group.

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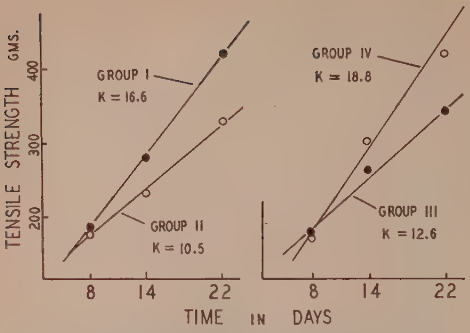

FIG. 1. Tensile strength plotted as function of time in days after wounding. The slope (K) is an index of the rate of healing.

wounds were made.§ The urea nitrogen and amino acid nitrogen returned to approximately the normal, prewounding levels within 5 days. This effect was found to be due to the anesthetic used and not to the wound.

In Fig. 1 is shown the rate of healing of the experimental wounds in terms of tensile strength and time. This can be expressed as, $T = \mathrm{K}\ t$, where T is the tensile strength in grams per 0.5 cm section of healing epithelium, t, the time in days after wounding, and K, the resulting slope. The slope, K, can be considered to be a measure of the rate of healing of the wounds. It will be seen that the rate of healing of the wounds in the animals fed the 6 percent casein diet (Group II; K = 10.5) is markedly less than that of the animals on the 20% casein diet (Group I; K = 16.6). This is in agreement with previous work which has indicated that wounds in animals on a high protein diet heal more rapidly than those in animals on a low protein diet(1-4). From this, it would be expected that the rate of healing of the wounds in animals in Group III should be the same as those in Group I, since the protein nitrogen intake in both groups was the same. Instead, the slope (K = 12.6) indicates that the rate of healing is very much less than that found in Group I. This difference in healing rate is obviously due to the very low biological value of the gelatin, supplemented to the diet of the animals in Group III to make it contain the same amount of protein nitrogen as is found in the diet of Group I.

Although the proteins ingested, and the protein nitrogen intake, is essentially the same in the animals comprising Groups III and IV, the rate of healing of Group IV (K = 18.8) is very much greater than that for the animals in Group III. This difference must be attributed to the methionine added to the diet of the rats in Group IV. It can then be concluded that the methionine increases the efficiency of utilization of dietary protein or tissue protein, or both. Since the methionine fed is probably available to the tissues for protein synthesis as cysteine and cystine, as well as methionine(19,20), all the sources of dietary sulfur might be considered together as "protein sulfur."

There appears to be no correlation between the protein nitrogen intake, or retention, and the tensile strength or rate of healing, when all 4 groups of animals are considered. The data in Table III, however, indicate a correlation between the amount of "protein sulfur" retained during the course of the experiment and the rate of healing of the wounds. It seems evident from these results that previous reports(1-8) on the effect of the level of protein in the diet on the rate of healing were indicative of the effect of the level of "protein sulfur" intake (and indirectly, the sulfur retention), rather than the effect of the protein itself. This is apparent from the fact that previous reports dealt with the effect of diets containing levels of the same protein; in which case, the sulfur content of the diets varied directly with the amount of protein in the diet.

The data in Tables II and III tend to indicate a larger proportion of sulfur to nitrogen is required during healing than for normal tissue synthesis. This is supported by the following: (a) after wounding, a positive sulfur balance is observed even when there is a negative nitrogen balance. This may mean that there is a sacrifice of tissue protein nitrogen so that the ratio of sulfur to nitrogen

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§ Data represent average urinary output of all animals in these experiments.

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TABLE III. Comparison of Avg Daily Intake and Retention of Dietary Nitrogen and Sulfur and Rate of Healing.*

Group #	Nitrogen: Avg daily intake, mg	Nitrogen: Avg daily balance, mg	Sulfur: Avg daily intake, mg	Sulfur: Avg daily balance, mg	Rate of healing index†
I	266	78	13.2	11.8	16.6
II	78	10	3.6	2.6	10.5
III	269	47	8.2	6.4	12.6
IV	251	56	23.1	14	18.8

* These values indicate daily avg for 21 days after wounding.
† See Fig. 1.

available is increased; (b) increasing the ratio of sulfur to nitrogen by dietary means (by the use of methionine) increases the rate of healing; (c) healing wound tissue contains larger amounts of sulfur than the same tissue when it has not been wounded(21,22).

Thus it seems probable that the rate of healing may be related to, among other factors, the amount of "protein sulfur" retained in excess over that utilized to form normal tissue protein with the retained nitrogen. Since the ratio of nitrogen to sulfur in most tissues is about 16:1, then the healing should be influenced by the amount of retained "protein sulfur," less 1/16 the quantity of retained nitrogen. This might be called the "excess sulfur" which is available for the greater demands of the healing wound tissue. The "excess sulfur" is 156, 44, 75 and 214 mg for the animals in Groups I to IV, respectively. Thus, there appears to be some correlation between the rate of healing and the "excess sulfur" available for the wound.

Summary. Wounded rats were kept on diets containing varying amounts of different proteins and "protein sulfur". There appears to be no relation between the amount of protein nitrogen fed or retained by the rats and the rate of healing. However, the "protein sulfur" retention appears to be correlated with the rate of healing. It is suggested that the amount of retained sulfur in excess over that utilized for normal tissue protein synthesis is an important factor in determining the rate of healing of experimental wounds.

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Variations in Proteolytic Activity of Serum of Animals Including Man.* (18760)

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In a previous publication(1) it was shown that there were significant differences in the activation of plasminogen to plasmin, by streptokinase and protamine, in seven species of animals. It was shown that in man only was there a significant increase of streptokinase activated plasmin over the spontaneously and protamine activated plasmin. In monkeys also there was probable though lesser activation by both streptokinase and protamine. It was also observed that the spontaneous activity in some animals was almost as great as the maximal activation by streptokinase in humans.

With this type of reaction it was necessary

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* This study was supported by the Damon Runyon Fund for Cancer Research, Institutional Research Grants 34 and 86.

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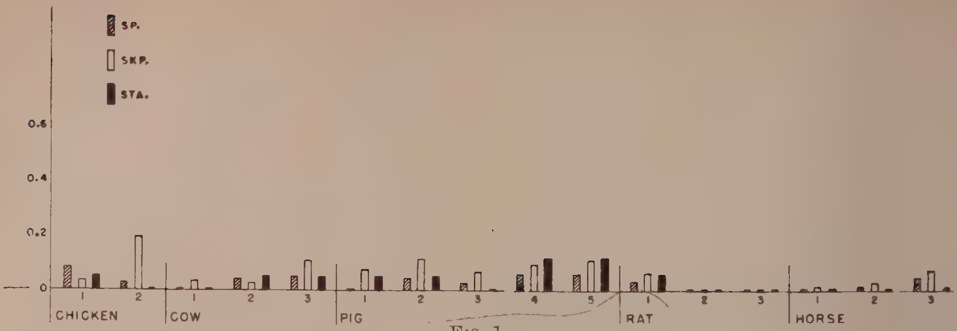

FIG. 1.

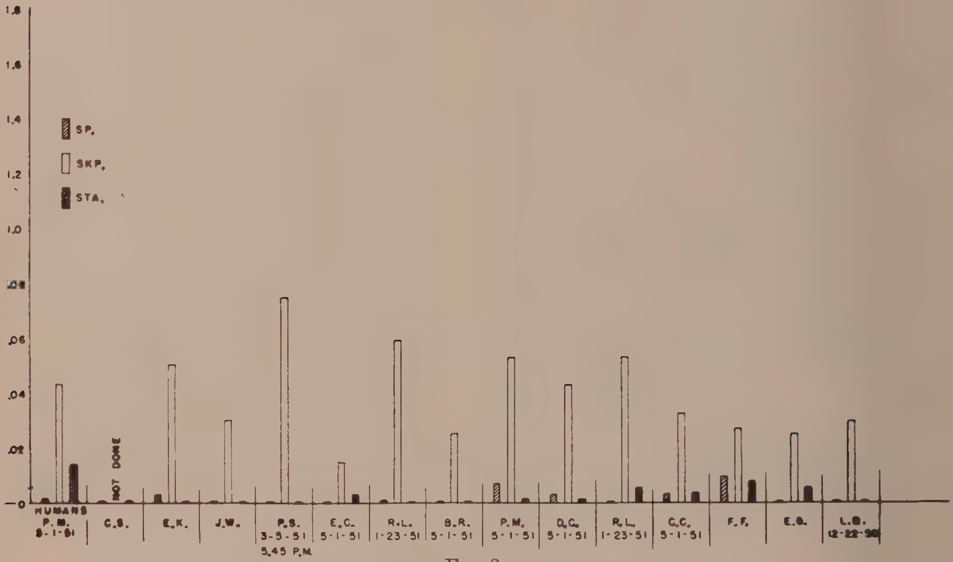

FIG. 2.

to do all activation studies with streptokinase on human serum which limited the experimental approach to the problem. Gerheim *et al.*(2) had previously reported the presence of a staphylokinase which also activated plasminogen(3), and in a later publication(4) they noted a variation in activation of animal sera by staphylokinase different from the activation pattern with streptokinase. They, however, used a fibrinolytic method of determination rather than the proteolytic (casein digestion) method. The present study was undertaken when an active staphylokinase was prepared according to the method of Gerheim, Ferguson, *et al.*(2).

Materials. 1. Plasminogen—prepared by the previously reported modification of Milstone's method(1). 2. Streptokinase (SKP) crude—the filtrate of a 24-hour culture of Group A, Type II, Beta hemolytic streptococci grown in beef heart infusion broth with 0.05% added dextrose. 3. Staphylokinase (STK)—prepared according to the method of

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2. Gerheim, E. B., Ferguson, J. H., and Travis, B. L., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 525.

3. Gerheim, E. B., Ferguson, J. H., Travis, B. L., Johnston, C. L., and Boyle, P. W., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 246.

4. Gerheim, E. B., and Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 261.

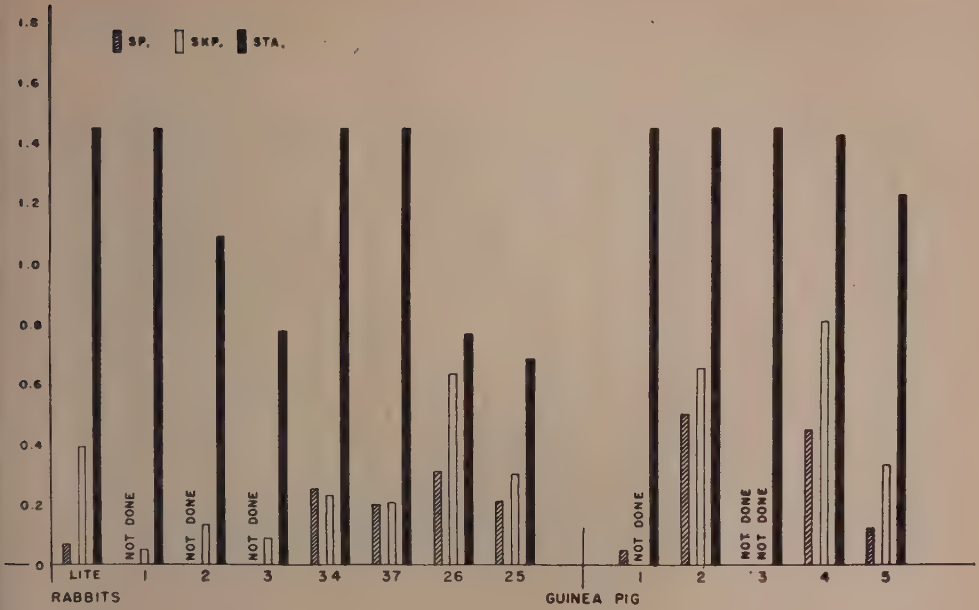

Fig. 3.

Ferguson† from a culture of a virulent *Staphylococcus aureus*(2). The remainder of the materials were the same as those used in the original method(1,5).

Method. As previously reported(5) except that the method of reading the result has been altered as follows: the blue dye, T-1824, is no longer used but a blank containing the reagents is run simultaneously. The color produced by the reagents used is then negated by setting the galvanometer index at 100% transmittance or at O concentration, with the blank cuvette in position. Using this as a reference, the per cent transmission of each aliquot is then read directly. Spontaneous activation and activation by both STK and SKP were tested in the following animals: 15 humans, 4 monkeys (*Macacus rhesus*), 9 dogs, 5 pigs, 3 cows, 3 horses, 8 rabbits, 3 guinea pigs, and 3 rats.

Results. A marked variation in the proteolytic activity of serum produced, as a result of the addition of STK to the euglobulin fraction, was observed in different animals (Fig. 1, 2, 3, and 4).

Horses, cows, pigs, and chickens showed very little activity either spontaneously or by action of SKP or STK (Fig. 1). Human patients once again showed a marked activation by SKP as with our previous series, but very little or no activation by STK except in one instance (P.M., Fig. 2). Even in this case the activation by SKP was 3 times as great as that by STK.

Rabbits, guinea pigs (Fig. 3), monkeys and dogs (Fig. 4) showed the maximum activation by STK with the readings actually off the colorimeter scale in most instances. Four of the 8 rabbits showed the maximum effect and the other 4 showed a marked STK effect. In general the SKP and spontaneous activities of the species were of a similar degree to that previously reported.

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† A small quantity of staphylokinase was originally supplied by the kindness of Dr. John H. Ferguson and Dr. Jessica H. Lewis of the Department of Physiology, University of North Carolina. This was used for preliminary studies not reported here. The staphylokinase used in these experiments was prepared in this laboratory with the help of Miss Arline Strouse of the Surgical Bacteriology laboratory.

5. Downie, G. R., and Cliffton, E. E., Proc. Soc. Exp. Biol. and Med., 1949, v71, 138.

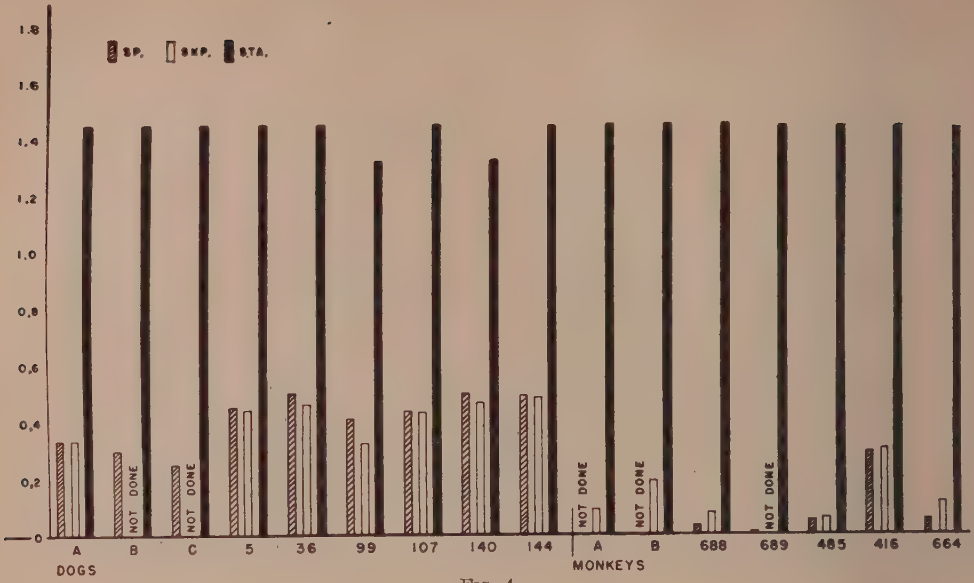

FIG. 4.

Discussion. The species variations in activation of plasminogen by SKP and STK are difficult to explain. One logical concept is that the animals which show no activation have active anti-kinases in their serum either as the result of low grade infections from these organisms or as a part of their normal armamentarium for defense. It is also possible that the plasminogen itself in these instances is resistant to the specific kinase. The present data with an increase of proteolytic activity with STK in dogs, rabbits, and guinea pigs disproves the previous supposition that these animal sera were not activated by SKP because the maximum amount of material available was spontaneously activated(5).

There is one variation from Gerheim and Ferguson's data(4); the lack of significant activation of human plasminogen by STK. However, this may well be due to the fact that they were using a fibrinolysin test and we were using a test of proteolytic activity (casein digestion). Preliminary studies indicate that there is a variation in fibrinolytic and proteolytic activity as produced by STK (data to be published).

The fact that STK activates the plasminogen from sera of the ordinary laboratory animals—monkeys, dogs, guinea pigs and rabbits—makes this an excellent tool for the further study of this enzyme system. Such study was previously markedly limited because SKP (the other rapid and fairly complete activator) activated only human serum plasminogen and not that of ordinary laboratory animals.

Summary. Plasminogen (euglobin fraction of serum) of certain animal species tested—dog, rabbit, guinea pig, and monkey—is activated by a staphylokinase obtained from a culture of virulent *Staphylococcus aureus.* Humans show very little activation with staphylokinase, but definite activation with streptokinase. The species activation pattern with staphylokinase and streptokinase varies markedly. Plasminogen from other animals (horse, cow, pig, and chicken) is activated very slightly, and some not at all, by any activator thus far employed.

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Measurement of "Extracellular Fluid Space" In Infants by Equilibration Technic Using Inulin and Sodium Ferrocyanide. (18761)

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"Extracellular fluid space" has been measured in adult man by Gaudino, *et al.*(1) and others(2) using inulin. These authors point out that inulin defines a smaller and probably more exact "extracellular space" than does thiocyanate, sucrose, mannitol, chloride, bromide, sulfate, and sodium. Levitt and Gaudino(3) in describing the method used in adults emphasize the need for complete urinary collection of the injected inulin in order to obtain reliable results. Using this technic 10 to 18 hours are required for the determination of the "extracellular space". To estimate the "extracellular fluid space" in sick infants it is desirable to employ a test which can be completed in a shorter period of time.

It would appear obvious that if one subtracted the amount of inulin excreted from the beginning of the injection to the end of equilibration period from the total amount given, the difference would represent the amount of inulin diffused throughout the "extracellular fluid space", and that from the estimation of the plasma level at this point one should be able to calculate the size of the "extracellular fluid space." Levitt and Gaudino(3) point out that this use of the equilibration period to estimate the inulin space would give unreliable results. This is not in agreement with the data presented below. The following data collected in 6 infants 9 days to 14 months of age with varying, severe congenital defects involving the central nervous system show a good correlation of "inulin" space when estimated both at the equilibration period and after collection of totally excreted inulin, thus making the test more applicable in the study of sick infants. In a group of these infants, sodium ferrocyanide at low plasma levels (5.5 to 12 mg %) was used simultaneously to measure the "extracellular fluid space". At these levels of plasma ferrocyanide (Table I) we found no urinary evidence of renal toxicity. However, Miller and Winkler(4) found such evidence with the use of sodium ferrocyanide in adults. In most of their cases the plasma level of ferrocyanide was higher than those obtained in our infants. This substance was chosen because of its smaller molecular size and with the thought that it would reach equilibration more rapidly than does inulin and therefore shorten the time necessary to measure the "extracellular fluid space". It may, therefore, be a more effective agent in measuring this space in the presence of edema. However, its exact toxicity is not established(5).

Methods. A 5% glucose solution containing 1% inulin and .01% sodium ferrocyanide is given as a constant intravenous infusion at the rate of 0.75 to 1.0 cc per minute. Equilibration was defined at the period when a constant plasma level was obtained. In these studies at least 3 identical plasma levels over a period of 30 to 45 minutes indicated that equilibration had taken place. Equilibration in these small infants was usually obtained for inulin within 2 hours and for sodium ferrocyanide within 45 minutes. The infusion was discontinued at the end of the equilibration period. Inulin was determined by Harrison's (6) modification of the method of Hubbard and Loomis(7). The ferrocyanide method of

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1. Gaudino, M., Schwartz, I. L., and Levitt, M. F., Proc. Soc. Exp. Biol. and Med., 1948, v68, 507.
2. Schwartz, I. L., Schachter, D., and Freinkel, N., *J. Clin. Invest.*, 1949, v28, 1117.
3. Levitt, M. F., and Gaudino, M., *Am. J. Med.*, 1950, v9, 208.
4. Miller, B. J., and Winkler, A., *J. Clin. Invest.*, 1936, v15, 489.
5. Stieglitz, E. J., and Knight, A. A., *J.A.M.A.*, 1934, v103, 1760.
6. Harrison, H. E., Proc. Soc. Exp. Biol. and Med., 1942, v49, 111.

TABLE I. Extracellular Fluid Space Measurements with Inulin and Sodium Ferrocyanide.

Patient	Age	wt, kg	Equilibration period, min.	Collection in hr after equil. per.	Method of determination	Total mg infused	Plasma level, mg %	Recovery, mg		% total recovery	Vol. of distribution as % body wt		Comment
								Equil. per.	Total		Equil. per.	Recovery	
L.	21 days	3.64	160	24	Inulin	1095	40.9	513	1066	99	37.8	37.4	Mental retardation
					Ferrocyanide	120	5.5	46.6	116.7	96.6	37.3	36	
F-1	9 ,,	3.19	275	22	Inulin	1635	62.3	1060	1607	99	29.1	28.8	Dehydrated spina bifida
F-2	30 ,,	3.28	168	26	Inulin	893	51.1	187.5	886.9	99	42.1	41.7	Hydrated spina bifida
					Ferrocyanide	98	6.2	14.5	95.5	97	42	40.7	
J-1	6 ,,	2.86	163	21	Inulin	1642	67.4	1000	1636	100	33.5	33.5	,, ,,
					Ferrocyanide	222.8	12	108	221.8	100	33.5	33.5	
J-2	34 ,,	3.04	132	22	Inulin	1325	81	575	1238	93.4	30.5	28.5	,, ,,
					Ferrocyanide	150.3	10.4	48.3	140.3	93.5	31.8	29.7	
S.	30 ,,	3.63	168	24	Inulin	1398	60.2	707	1330	95.2	31.6	30	,, ,,
					Ferrocyanide	145.4	7.8	58.8	137.4	95	30.5	29	
K.	5 Mo.	7.60	180	24	Inulin	2424	38	1630	2421	100	27.5	27.5	Cerebral porencephaly
C.	14 ,,	6.40	220	21	Inulin	3759	31.7	3050	3782	100.6	36.1	36.1	Men. deficiency

Husson(8) adapted for the measurement of sodium ferrocyanide at low concentrations in body fluids was used.* For the precise estimation of the inulin and sodium ferrocyanide injected, a sterile 500 cc volumetric flask was used as a reservoir from which an aliquot was infused by means of an open burette flask. After discontinuance of the infusion, the remainder of the solution in the infusion system including the tubing was collected into the reservoir volumetric flask. We have found in the infusion system adhering to its walls after drainage as much as 169 mg of inulin and 5 mg of sodium ferrocyanide. If these materials are not carefully recovered, large errors in "space" measurement will be obtained. By subtracting the total residue of inulin or sodium ferrocyanide from the original, one obtains the amount given the patient.

Results. Table I is a summary of the data collected in 8 tests performed in 6 individual infants. It shows a close agreement between the inulin "extracellular fluid space" estimated at the equilibration period and again with "complete" urinary collection of inulin over a period ranging from 21 to 26 hours. The low value for "extracellular fluid space" found in case F-1 at the age of 9 days is explained by the existence of dehydration at that time. Similarly case J-2 was slightly dehydrated as a result of diarrhea when the test was performed at 34 days of age.

In 4 of these infants 5 simultaneous measurements of ferrocyanide space showed close agreement with the inulin space both at the equilibration period and after "complete" urinary collection of ferrocyanide. The equilibration period for sodium ferrocyanide was intentionally prolonged until the end of the inulin equilibration period in order to compare the results with these two substances under identical circumstances. The high percentage

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7. Hubbard, R. S., and Loomis, T. A., *J. Biol. Chem.*, 1942, v145, 641.

8. Husson, G. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 230.

* In preparation of the filtrate 1 cc of the serum, urine and standard solution are used and are diluted with 3 cc of water, 4 cc acid mixture and 1 cc 20% trichloroacetic acid. Four cc aliquots of these filtrates are used for the determinations.

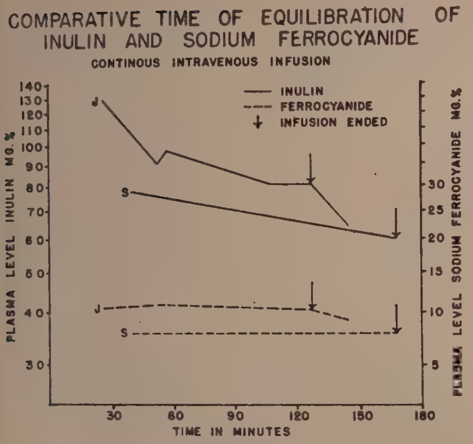

FIG. 1.

COMPARATIVE CLEARANCES OF INULIN AND SODIUM FERROCYANIDE

PATIENT	GLOMERULAR FILTRATION RATE CC./MIN. (UNCOR.)		RATIO NA FE/INULIN
	INULIN	NA FERROCY.	
J.			
PERIOD 1	5.5	4.7	0.85
" 2	5.1	4.3	0.84
" 3	4.7	3.9	0.83
S.			
PERIOD 1	7.7	6.4	0.83
" 2	7.8	6.4	0.82
" 3	6.1	5.1	0.83

FIG. 2.

of recovery of both inulin and ferrocyanide attests the validity of space estimation at the equilibration period. The close correlation of space estimation between these two periods for both substances would suggest that the renal "deadspace" in these infants is not a significant factor in estimating "extracellular fluid space".

Fig. 1 shows the relative rates at which inulin and ferrocyanide reach equilibration in 2 infants. The infusions were begun at zero-time and stopped at the time shown by the arrows on the graph. It is apparent that ferrocyanide reaches equilibrium much sooner than does inulin, which might have been expected from its smaller molecular weight, and that the "extracellular fluid space" could be determined more quickly using ferrocyanide than inulin.

Fig. 2 shows comparative rates of glomerular filtration when continuous infusions of inulin and ferrocyanide were given simultaneously in 2 of these infants. The plasma concentrations of arterial blood were used in calculating these clearance values. The inulin clearances are within normal values for the age group(9). The lower clearance values obtained with ferrocyanide, giving a ratio of Fe/In of 0.82 to 0.85, would indicate tubular reabsorption of ferrocyanide as was found in the adult by Miller and Winkler(4) who demonstrated that the ferrocyanide clearance was of the same order of magnitude as the urea clearance.

Summary. A close correlation of "extracellular fluid volume" as measured at equilibration and after collection of totally excreted inulin and sodium ferrocyanide was obtained in infants, thus shortening considerably the time needed for the determination. The ratios between the sodium ferrocyanide and inulin spaces were 0.96 to 1.04. Sodium ferrocyanide equilibrated in a much shorter time than did inulin. The comparative rate of glomerular filtration as measured by inulin and sodium ferrocyanide suggests tubular reabsorption of the latter substance in infants. No evidence of urinary disturbance was found in the infants given sodium ferrocyanide.

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9. Rubin, M. I., Bruck, E,. and Rapoport, M., *J. Clin. Invest.*, 1949, v28, 1144.

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Relationship of Pyridoxine Deficiency to Adrenal Function in Production of Leucocytes in Mice.* (18762)

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Granulocytosis, lymphopenia, and myeloid metaplasia in the spleen have been produced in mice either by treatment with cortisone or by the production of pyridoxine deficiency induced with desoxypyridoxine(1,2). This similarity of effect suggested a common mechanism of action. The possibility that pyridoxine deficiency induced by desoxypyridoxine merely activated an alarm reaction, mediated through the adrenal, was considered, although significant differences in the hematologic pictures produced by the 2 methods were pointed out. The results of 3 other experiments reported here support the view that the mechanism responsible for the production of the hematologic abnormalities in pyridoxine deficiency in the mouse is independent of the adrenal gland.

Exp. 1. 15 weanling Swiss mice weighing within 7 g of one another were placed in individual cages and divided into 3 groups of 5 each. Group I was fed ground Friskies dog chow to which was added 0.1% desoxypyridoxine HCl. Group II was fed dog chow and given 1 mg of cortisone subcutaneously daily. Group III received both cortisone and desoxypyridoxine. At the beginning of the experiment and twice weekly thereafter total and differential leucocyte counts were done and the animals weighed. Sections of spleen, liver, bone marrow, adrenal glands, and lymph nodes were obtained at autopsy. The simultaneous induction of pyridoxine deficiency and administration of cortisone apparently produced greater changes in the circulating neutrophiles and lymphocytes than either alone (Fig. 1). The leucocyte shift was more complete on the 4th day and the lymphopenia was more severe with earlier mortality and greater weight loss. Histologic changes in the organs were the same as described before(2) and were not significantly different in the 3 groups. The greater effect produced by pyridoxine deficiency plus cortisone as compared to the effect of either alone indicated that the mechanism of action of the one may differ from the other.

Exp. 2. Bilateral adrenalectomy through a single dorsal incision was done on 22 weanling Swiss mice. The entire group was given 0.85% salt solution for drinking water and a diet of Friskies dog chow. They were divided into 2 groups of 11 each. The first group received 0.1% desoxypyridoxine added to the diet. The other group served as controls. On the 15th day, after hematologic changes had occurred, 0.3% pyridoxine HCl was added to the diet of all the survivors. The leucocyte changes in the adrenalectomized mice given desoxypyridoxine (Fig. 2) were identical with those occurring in intact mice given desoxypyridoxine, as previously reported(1). Absolute neutrophilia and lymphopenia occurred which was reversed by the addition of pyridoxine to the diet. Although 7 of the 11 mice receiving desoxypyridoxine died in the first week, hematologic changes had already occurred. Only 2 of this group survived the entire experimental period. There was no significant change in the leucocyte counts of the control group of 11 adrenalectomized mice not given desoxypyridoxine (Fig. 3). Although the lymphocyte counts increased moderately, they remained within normal limits. Only 2 mice had died at the end of the first week and 4 survived the experimental period. Histologic sections from animals which died before pyridoxine was added to the diet were unsatisfactory because of post-mortem degeneration. In the spleens of animals killed at the end of the experiment there was a slight, but

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* These studies were supported by a grant from the National Vitamin Foundation.

† American Cancer Society Clinical Fellow.

1. Weir, D. R., Heinle, R. W., and Welch, A. D., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 457.

2. Weir, D. R., and Heinle, R. W., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 655.

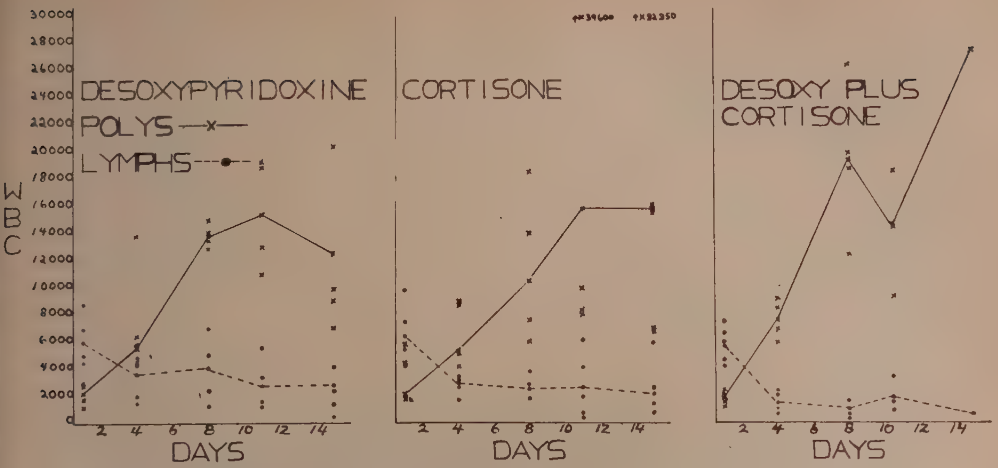

FIG. 1. Effect on circulating leucocytes of cortisone, of pyridoxine deficiency, and of the two combined.

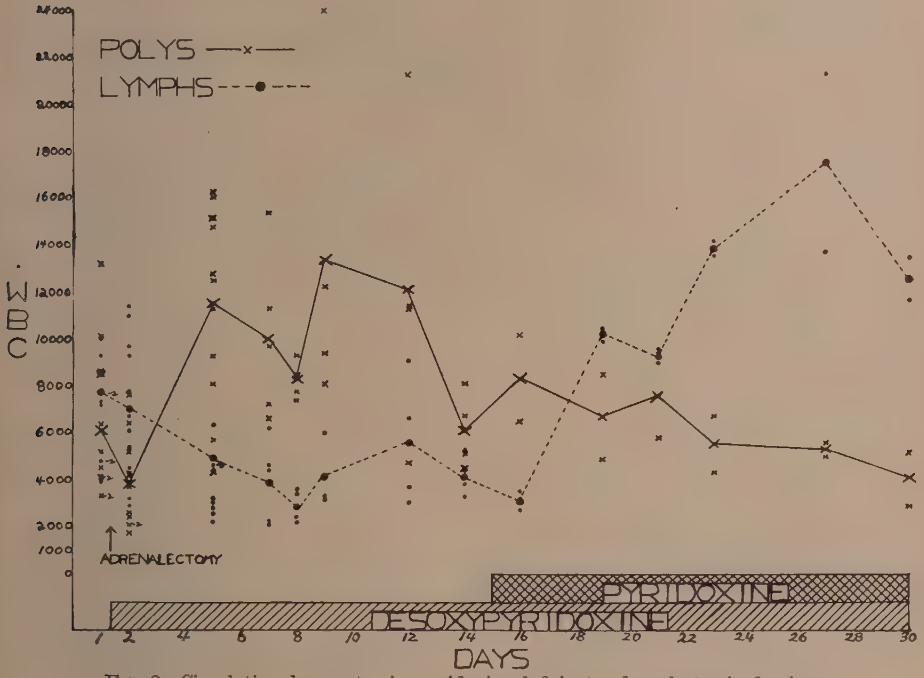

FIG. 2. Circulating leucocytes in pyridoxine-deficient, adrenalectomized mice.

definite, increase in the size, number, and activity of the follicles, with increased numbers of nucleated red cells in the pulp. The fact that adrenalectomy failed to prevent the leucocyte changes caused by pyridoxine deficiency is clear evidence that the mechanism of production of these changes is not mediated through the adrenal.

Exp. 3. It was suggested previously(2) that one of the effects of cortisone might be

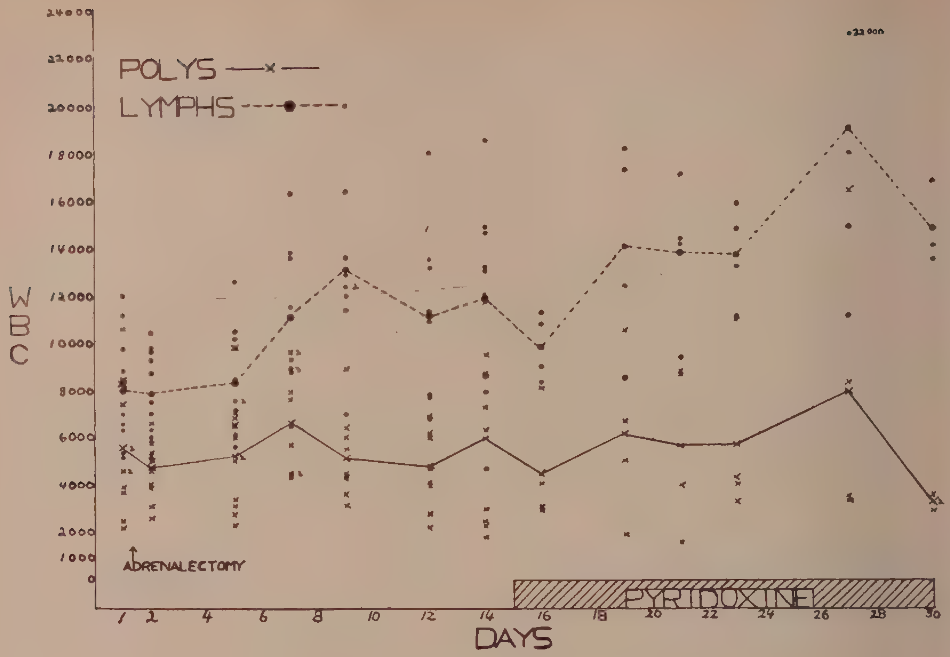

FIG. 3. Circulating leucocytes in untreated, adrenalectomized mice.

the induction of an effective deficiency of the active vit. B_6 coenzyme, occurring at some stage in the metabolic cycle in which the vitamin is concerned. In order to obtain evidence for or against this hypothesis an attempt was made to block the hematologic effect of cortisone by feeding large quantities of the B_6 vitamins to cortisone-treated mice. A diet was prepared containing 0.3% each of pyridoxine HCl, pyridoxal HCl, and pyridoxamine dihydrochloride added to ground Friskies dog chow. Since a mouse eats 3 to 6 g of diet daily, this formula provided 9 to 18 mg of each metabolite to each mouse daily. Twenty Swiss mice were divided into 4 groups of 5 each. Group 1: B_6 diet and 1 mg cortisone. Group 2: B_6 diet and 0.5 mg cortisone. Group 3: normal diet and 1 mg cortisone. Group 4: normal diet and 0.5 mg cortisone. Cortisone was given subcutaneously daily. Groups 1 and 2 were maintained on the supplemented diet for 8 days before cortisone was started.

The animals eating the normal diet developed the expected neutrophilia and lymphopenia. These changes were more marked in the animals receiving 1 mg of cortisone than in those receiving 0.5 mg. There was no evidence that the hematologic response to cortisone was in any way modified by the added B_6 vitamins in Groups 1 and 2. In spite of the enormous doses of the vitamins the findings were entirely comparable with those of the control groups. This may indicate that the mechanism for the production of hematologic changes induced by cortisone differs from that of pyridoxine deficiency but the possibility still exists that cortisone may have a role in the B_6 metabolic cycle, perhaps by blocking the production or utilization of pyridoxal phosphate or pyridoxamine phosphate.

Discussion. It has been shown(2) that in pyridoxine deficiency induced by desoxypyridoxine the hematologic changes can be maintained for at least 30 days, a result not characteristic of the alarm reaction, in which there is a reversal towards normal in a brief period of time. Also, the fact that the hematologic picture can be restored to normal by the addition of pyridoxine indicates that the effect of desoxypyridoxine is probably that of a metabolic competitor to the active B_6 vitamin and, in itself, does not activate an alarm reaction. The demonstration that the hematologic abnormalities occur in pyridoxine-deficient, adrenalectomized mice proves conclusively that the mechanism is not that of an alarm reaction mediated through the adrenal. Thus, the mechanism for the production of these abnormalities remains to be elucidated. It may conceivably involve interference with some enzyme activity in nucleoprotein metabolism not understood at this time.

Since simultaneous pyridoxine deficiency and cortisone administration produced greater changes than either alone, the possibility should be considered that cortisone actually accelerated the production of pyridoxine deficiency. Stoerk(3) considered this when he observed that the combination was more deadly in rats than either one alone. He offered the hypothesis that cortisone, by increasing the utilization of amino acids, increased the body's demand for vitamin B_6 and thereby further aggravated the pyridoxine deficiency. Against this hypothesis is our finding that enormous amounts of pyridoxine, pyridoxal, and pyridoxamine in no way modified the hematologic effect of cortisone. Since the hematologic changes are so similar in these two states, the possibility exists, however, that the ultimate mechanism may be the same. Thus, the effect might be on the "end-organ" whether this be nucleoprotein synthesis or some other unknown metabolic reaction. This would adequately explain all the results of these experiments.

Summary. 1. The production of pyridoxine deficiency in adrenalectomized mice calls forth the same hematologic response, neutrophilia and lymphopenia, as in intact animals. This is positive evidence that these blood changes occurring in pyridoxine deficiency are not the result of an alarm reaction mediated through the adrenal gland. (2) Pyridoxine deficiency and cortisone administration produce similar hematologic responses. When combined, a synergistic effect results. This may be evidence that the mechanism of the production of the hematologic changes is different in the 2 conditions. (3) Further evidence that the mechanisms may differ is the fact that the cortisone effect cannot be blocked by giving large amounts of pyridoxine, pyridoxal, and pyridoxamine, although the possibility exists that cortisone has a role at another site in the B_6 cycle.

Pyridoxine, pyridoxal, pyridoxamine, and desoxypyridoxine were supplied by Merck and Co.

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Interrelationships of Folic Acid and Ascorbic Acid in Rat Liver.* (18763)

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Nichol and Welch(1,2) reported that one probable role of ascorbic acid in animal tissues is to aid in the conversion of folic acid to the *Leuconostoc citrovorum* factor (LCF). Recently we have reported that the choline oxidase system of rat tissues is intimately related to folic acid, ascorbic acid, LCF, and vit. B_{12}(3,4) as demonstrated by *in vitro*

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3. Williams, Jr., J. N., *J. Biol. Chem.*, 1951, v190, in press.

experiments. When rats are made folic acid "deficient" by feeding aminopterin, the choline oxidase activity of the liver is decreased considerably. The activity may be increased to values approaching normal by the incubation *in vitro* of the factors just named with the rat liver homogenate prior to addition of the substrate. Some of the factors, *i.e.*, ascorbic acid and folic acid, also stimulate normal rat liver choline oxidase, though not as markedly as the liver enzyme from aminopterin-fed rats. Since the most active factor for stimulating choline oxidase activity *in vitro* was ascorbic acid(4), it appeared possible that one of the reasons for the lowered choline oxidase activity in aminopterin-fed rats was a decreased synthesis of ascorbic acid by these rats, as well as a blocking of the conversion of folic acid to the *L. citrovorum* factor.

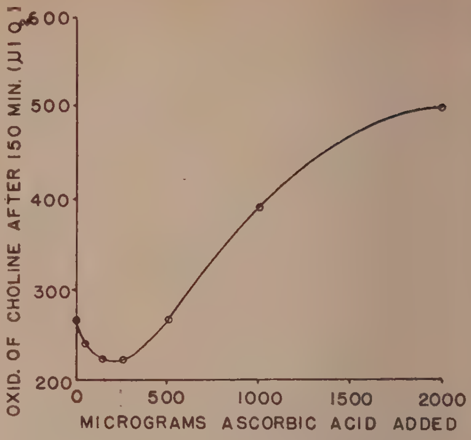

Fig. 1. Effect of ascorbic acid added *in vitro* to a normal rat liver homogenate upon oxidation of choline in the presence of homocysteine.

Experimental. Adult male rats of the Holtzman strain weighing from 200 to 250 g were used as experimental animals. They were divided into 2 groups of 7 animals each. One group received a purified ration containing 18% casein as previously described(1), and the other group was fed the same ration with 4 mg aminopterin† per kilo ration. The animals fed aminopterin developed marked deficiency symptoms within about 10 days. They were used in the analytical or enzyme studies immediately after the first noticeable symptoms developed. The analyses for ascorbic acid content of the livers of both groups of rats were carried out by titration with 2,6-dichlorophenolendophenol reagent. In several cases a check on this method was made using the colorimetric method of Roe and Oesterling(5) as modified by Bolomey and Kemmerer(6) and Bolin and Book(7). In the latter determinations values for reduced, oxidized, and total ascorbic acid were obtained. In the titration method 1 part of liver was homogenized in 5 parts of water, and 3 ml of 30% trichloroacetic acid were added to 10 ml of the homogenate. After centrifuging, 4 ml of the supernatant extract was titrated with 0.025% 2,6-dichlorophenolendophenol reagent. In the colorimetric method 1 g of liver was homogenized in 4 ml mixed acid(7) and diluted to 20 ml with mixed acid. After centrifuging and filtering, the ascorbic acid of the extract (total, reduced, and oxidized) was determined according to the references listed above.

In the enzyme determinations the effect of various levels of ascorbic acid added *in vitro* to the liver homogenates of normal rats was studied. The enzyme system and method of assay have been described previously(4). In the present experiments the levels of neutralized ascorbic acid given in Fig. 1 were added to one side-arm of a series of double side-armed flasks at the expense of water. After incubating the ascorbic acid with the liver homogenate, the choline and homocysteine were added from the second side-arm, and readings were taken at 10-minute intervals for 150 minutes. Oxygen uptake due to oxidation of choline was obtained by subtracting the endogenous oxygen uptake in the flask without added choline from the flask containing choline.

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† The author wishes to thank Dr. J. J. Oleson of Lederle Laboratories, Pearl River, N. Y., for the generous supply of aminopterin used in these studies.

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Results. The ascorbic acid determinations with the 2,6-dichlorophenolendophenol reagent in the livers of the 2 groups of rats gave the following results: Rats fed the complete basal, 403 ± 27[‡] γ per g liver; rats fed the complete basal + aminopterin, 189 ± 20[‡] γ per g liver. The colorimetric results checked very well with the titration results and in no instance was any measurable oxidized ascorbic acid found in the livers of either groups of rats. From the results it can be observed that the livers of the rats fed aminopterin exhibit a considerably lower ascorbic acid concentration than the livers of rats fed the same ration without aminopterin. Therefore, it appears that aminopterin not only blocks the utilization of folic acid but also blocks the endogenous synthesis of ascorbic acid in the rat and that folic acid (or *L. citrovorum* factor) is probably involved in the synthesis of ascorbic acid as well as in other functions. If the utilization of folic acid is inhibited by aminopterin, the synthesis of ascorbic acid, which incidentally is also involved in the utilization of folic acid itself(1,2), is inhibited.

In Fig. 1 are presented the results of the effect of various levels of ascorbic acid on choline oxidase activity in the presence of homocysteine. It should be emphasized here that these effects and those presented in an earlier paper(4) can be demonstrated best in the presence of homocysteine, though less amplified effects can be observed without homocysteine(3). The results in the figure are presented as total oxidation after 150 minutes due to added choline (μl of O_2) versus the level of ascorbic acid (γ) added to the flasks. The results in the figure depict a typical experiment with a normal rat fed the complete synthetic ration. It appears that low levels of ascorbic acid added *in vitro* actually inhibit the oxidation of choline in the system employed while higher levels (over 500 γ per flask) stimulate the system markedly. This unique situation is difficult to explain from present knowledge. It may, however, be related to the decreased choline oxidase activity observed in tissues of animals fed aminopterin. Since as shown above the concentration of liver ascorbic acid is markedly decreased by dietary aminopterin and since low levels of ascorbic acid inhibit choline oxidase rather than stimulate it, the low liver ascorbic acid concentrations of rats fed aminopterin may help to explain the low choline oxidase activity of livers of those rats.

The fact that all of the ascorbic acid present in the livers of both groups of animals is reduced indicates that the action of the aminopterin is to block the actual synthesis of the ascorbic acid molecule rather than merely to inhibit maintenance of the reduced form. Further studies need to be made to discover the points in ascorbic acid synthesis which are blocked by aminopterin or in which folic acid is involved.

Summary. 1. Rats fed aminopterin exhibit a concentration of liver ascorbic acid less than one-half normal, indicating that folic acid is probably involved in ascorbic acid synthesis in the rat. 2. Enzyme studies indicate that low ascorbic acid levels added *in vitro* reduce liver choline oxidase activity in the presence of homocysteine whereas higher levels stimulate activity of the enzyme system. Implications of this phenomenon with depression of choline oxidase activity by dietary aminopterin have been discussed.

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[‡] Standard error of the mean.

Ascorbic Acid and the Hyaluronidase Hyaluronic Acid Reaction. (18764)

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(Introduced by Smith Freeman.)

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The histologic studies of Wolbach and Howe(1) showed the antiscorbutic value of vitamin C in scurvy to be due to its effect upon the formation of intercellular cement substance, and it has been generally assumed that a similar action explains its effects in cases of increased capillary fragility corrected by vitamin C. However, the recent work of Hines and Parker(2) showed that large doses of vitamin C given intravenously could correct increased capillary fragility in some patients within a period of 10-30 minutes, indicates an additional action of the vitamin. The rapidity of this action suggests a direct chemical or enzymatic response and since the spreading factor, hyaluronidase, has been shown to increase capillary permeability(3), the present studies were carried out to investigate possible relationship of ascorbic acid to the hyaluronidase-hyaluronic acid system. Three methods were used for the study:

Experiment 1. Experiments were first made to study the *in vitro* effect of vit. C on the hyaluronidase-hyaluronic acid reaction. For this purpose an impure solution of hyaluronic acid was obtained by filtering fresh vitreous humor from bovine eyes. One cc of this solution was mixed with an equal volume of serum and the amount of hyaluronidase* which would prevent precipitation of the hyaluronic acid by dilute acetic acid was determined(4), (the reaction was carried out at 37°C for 20 minutes with pH adjusted to 7.5). This amount of hyaluronidase was then mixed with the substrate which consisted of a serum mixture containing varying dilutions of buffered vit. C. (Simultaneous controls were run using normal saline in place of vit. C). The effects on turbidity were measured in a photolometer.

Experiment 2. A standard quantity of hyaluronidase colored with phyloxine dye, and brought to constant volume (0.5 cc) with isotonic sodium chloride was injected into the skin of the shaved abdomen of a rabbit and the amount of spread at the end of 20 minutes measured. The area of spread was calculated according to the formula for the area of an ellipse ($A = \frac{Dxd}{4}$, where $D =$ greatest diameter of spread and $d =$ smallest diameter of spread)(5). Having determined the area of spread of the control injections of hyaluronidase, the same quantity of enzyme was then injected with varying amounts of ascorbic acid while a constant volume of each injection was maintained by varying amounts of sodium chloride. The area of spread in 20 minutes was calculated by the above formula.

Experiment 3. In this set of experiments, the area of spread of the control solution of hyaluronidase was measured before, and at 10-minute intervals after, the intravenous injection of 500 mg of vit. C. Blood vit. C levels were also determined at varying intervals.

Results. The *in vitro* tests showed that slight inhibition of the hyaluronidase action was produced by ascorbic acid. The greatest

TABLE I. Effect of Mixtures of Ascorbic Acid and Hyaluronidase on Area of Spread.*

	D	d	Avg area of spread
Control tests	3.18 cm	2.36 cm	5.82 cm^2
Tests with ascorbic acid	1.25	.85	.79

* Three rabbits were each tested 3 times in the absence of ascorbic acid and 5 times with ascorbic acid.

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* The hyaluronidase used in this study was obtained through the courtesy of Wyeth, Inc.

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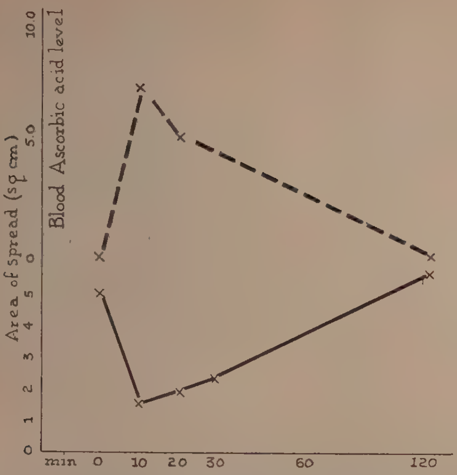

FIG. 1. Effect of intravenous ascorbic acid on capillary permeability "hyaluronidase spread."

inhibition was noted with 0.01 mg of ascorbic acid, but such inhibition was only 7% complete.

The local inhibitory effect of ascorbic acid on "hyaluronidase spread" in the rabbits' skin was much more marked. The area of spread of the control injections averaged† 5.82 sq. cm, but when ascorbic acid in amounts varying from 5 to 50 mg was added the area of spread averaged only 0.79 sq. cm (Table I).

The inhibitory effect of ascorbic acid on "hyaluronidase spread" was also quite marked when it was given intravenously to the animal. The average area of spread of hyaluronidase before injection of ascorbic acid was 5.43 sq. cm; following the injection of ascorbic acid the average area of spread was reduced to a low of 1.51, 10 minutes after the injection. This effect and the ascorbic acid blood levels are shown in Fig. 1.

Discussion. The structure of the capillary wall has been discussed by Chambers and Zweifach(4), who showed that there are 3 important components. The capillary endothelium is apparently not a factor in capillary permeability. However, the endothelial cells probably do secrete the intercellular cement, and alterations in this cement will result in permeability changes. The second important component of the capillary is the endocapillary layer, which is considered to be a coating of the endothelial cells, formed by absorption of blood proteins. Changes in the endocapillary layer may also result in increased capillary permeability. A third component of the capillary is the pericapillary sheath, composed of connective tissue, which surrounds the capillary vessels.

It is apparent that alteration in any one component of the capillary wall could result in increased permeability. The location of the defect corrected by ascorbic acid has not been determined. Extensive studies by Wolbach(1,7,8) have shown that in the absence of ascorbic acid normal cement substance is not produced. This has been confirmed by Dalldorf(9) who showed that scorbutic animals, when treated by vit. C, will produce normal cement substance in approximately 18-24 hours. However, in the capillaries, no morphologic changes have been seen, and whether the deficiency in capillary permeability is in the cement substance or in the pericapillary sheath has not been determined.

The exact composition of the cement substance is also not known and it is unknown whether or not it contains hyaluronic acid. Chambers and Zweifach(6) claim that hyaluronidase has no effect upon the cement substance and this indicates that it probably is not hyaluronic acid. Meyer(10), also contends that hyaluronic acid is not present in the cement substance. The pericapillary sheath, however, is composed of connective tissue and as such contains hyaluronic acid. It is probably this component of the capillary wall which is broken down when hyaluronidase is added, and causes increased permeability. It would seem, therefore, from the present studies, that the rapid action of ascorbic acid in some cases of capillary fragility is due to its effect upon the hyaluronidase-hyaluronic acid reaction in the pericapillary sheath rather than to an effect upon the intercellular cement substance. This is suggested because the action is rapid and does not take place in the 18- to 24-hour period characteristic of action on the cement substance. Ascorbic acid either inhibits hyaluronidase directly, or it alters the hyaluronic acid in the sheath so as to make it more resistant to hyaluronidase.

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† In this and the following set of experiments, 3 rabbits with 8-10 injections each were used and the results averaged.

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Summary. Both *in vitro* and *in vivo* experiments show that ascorbic acid exerts an inhibitory effect upon the hyaluronidase-hyaluronic acid reaction. The inhibition is more marked in the animal than in the test tube.

The components of the capillary wall and their relation to capillary permeability have been discussed.

These studies suggest that in some cases where capillary fragility is corrected by ascorbic acid, the effect could be due to the inhibitory effect of ascorbic acid upon a hyaluronidase-hyaluronic acid system, rather than to an effect of vit. C on the intercellular cement.

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Some Hormones Involved in Elaboration or Activation of the Mammary Spreading Factor.* (18765)

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In a previous paper(1) observations were presented indicating the presence of a spreading factor in the mammary glands of pregnant albino rats. This factor, which increased in potency during the first half of pregnancy, is thought to make possible the rapid advance of the mammary gland into the subcutaneous tissue. The close relation of mammary gland growth to the amount of active spreading factor suggested that the hormones regulating mammary gland growth might also regulate elaboration or activation of the spreading factor.

Hormones involved in mammary gland growth have been adequately reviewed by Turner(2), Riddle(3), Folley and Mallpress (4), and Trentin and Turner(5), so no attempt at review will be made.

Methods and materials. Castrate male and female albino rats weighing from 150 to 250 g were employed. If only castrate animals were to be used treatment was started on the eleventh day postoperatively, however, some castrate-adrenalectomized rats were used. In these cases the castrate animals were adrenalectomized on the eleventh day and treatment was started about 5 days later. The castrate-adrenalectomized animals were maintained on 1% sodium chloride drinking water. The estradiol benzoate and progesterone used in the treatment were dissolved in olive oil, the testosterone was dissolved in 60% alcohol, while the anterior pituitary extract† was made up as a suspension in distilled water. The treatment involved subcutaneous injections daily for 10 days. On the eleventh day the animals were killed, the mammary glands re-

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* Contribution from the Department of Dairy Husbandry, Mo. Agric. Exp. Station, Journal Series No. 1257 Approved by Director of Missouri Agric. Exp. Station.

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† The preparation employed was an ant. pituitary extr. rich in lactogenic hormone. This extract was generously supplied by United Research Laboratories, Philadelphia, Pa.

TABLE I. Effects of Hormone Treatment on Amount of Spreading Factor.

Controls & treatment	No. of animals and sex	Assay of spreading factors* Avg initial bleb, mm	Avg final bleb, mm	Area of spread, mm²
pH 6 buffer solution	34 trials	14 × 14	16 × 17	59.7
Testicular extract	34 ”	”	24 × 25	317.4
12 days pregnant	27 ♀	13.9 × 14	22.6 × 23.5	264.3
1 μg estradiol benzoate	5 ♀ †	14 × 14	16 × 18	72.3
3	5 ♀ †	13 × 14	19 × 20	155.6
5	5 ♀ †	14 × 14	20 × 21	176
5	5 ♂ †	”	20 × 21	176
5	5 ♀ † adren.	”	16 × 17	59.7
1 mg progesterone	5 ♀ †	14 × 14	16 × 18	72.3
3	5 ♀ †	13 × 14	17 × 18	97.4
5	5 ♀ †	14 × 14	18 × 19	114.7
5	5 ♂ †	”	18 × 18	100.6
5	5 ♀ † ”	”	18 × 19	114.7
10	5 ♀ †	”	21 × 21	192.5
10	5 ♂ †	13 × 14	20 × 21	187
10	5 ♀ † ”	14 × 14	21 × 21	192.5
1 μg E. B. + 1 mg prog.	5 ♀ †	13 × 14	16 × 16	58.1
1 3	5 ♀ †	14 × 14	20 × 21	176
1 5	5 ♀ †	”	21 × 22	209
1 5	5 ♀ † adren.	”	21 × 22	209
3 1	5 ♀ †	”	17 × 17	73.1
3 3	5 ♀ †	”	20 × 21	176
3 5	5 ♀ †	13 × 14	20 × 21	187
5 1	5 ♀ †	14 × 14	18 × 19	114.7
5 3	5 ♀ †	”	18 × 18	100.6
5 5	5 ♀ †	”	19 × 19	129.6
5 5	5 ♀ † adren.	”	18 × 19	114.7
1 mg testosterone	5 ♀ †	”	19 × 20	144.6
1	5 ♂ †	”	19 × 21	159.5
1	5 ♂ † adren.	”	19 × 20	144.6
10 mg A. P. extract	5 ♀ †	”	18 × 19	114.7
10 ” ” ” + 1 μg E. B.	10 ♀ †	”	21 × 21	192.5

* .2 extract + .1 cc Evans blue dye. † Castrate animal. Prog. = progesterone. Adren. = adrenalectomized. E. B. = Estradiol benzoate. A. P. = Ant. pituitary.

moved, extracts made and the amount of spreading factor in the extracts determined by a method previously described(1).

Experimental. The first experiments involved the injections of 1, 3 and 5 μg of estradiol benzoate. The second experiment employed the injection of 1, 3, 5 and 10 mg of progesterone. Experiments were also completed with various combinations of 1, 3 and 5 μg of estradiol benzoate and 1, 3 and 5 mg of progesterone. One mg of testosterone was injected into 5 castrate females, 5 castrate males and 5 castrate-adrenalectomized males. A 10 mg level of anterior pituitary extract was injected alone into five castrate females and together with 1 μg of estradiol benzoate into 10 castrate females.

Results. The spreading properties of the mammary gland extracts from the hormone injected rats were compared to testicular extracts and pH 6.0 buffer solutions (Table I).

The extracts from the 1 μg estradiol benzoate injected castrate females showed the presence of very little spreading factor. When the estradiol benzoate level was raised to 3 μg the mammary gland extracts indicated good elaboration or activation of the spreading factor. Mammary gland extracts from castrate males and females treated with 5 μg of estradiol benzoate indicated the presence of slightly more spreading factors. However, the mammary gland extracts from castrate-adrenalectomized females injected with the above level of hormone indicated the presence of no active spreading factor.

With progesterone alone the amount of spreading factor in the mammary gland extracts was small at the 1 mg level. More spreading factor was demonstrated with increasing amounts of hormone. The extracts from castrate males, castrate females and castrate-adrenalectomized females indicated the presence of fair amounts of the spreading factor at the 5 mg level and a further increase when the hormone level was doubled.

The combination of 1 μg of estradiol benzoate with 1, 3, and 5 mg of progesterone gave increasingly higher amounts of the spreading factor.

When the amount of estradiol benzoate was raised to 3 μg in combination with 1, 3 and 5 mg of progesterone the response was not quite as good but showed the same graded effect. When the estradiol benzoate level was raised to 5 μg with the above amounts of progesterone uniformly smaller amounts of the spreading factor were shown to be present in the mammary gland extracts of castrate animals. In a group of castrate-adrenalectomized females on this same level of hormone, the amount of spreading factor was about the same. Mammary gland extracts from the various groups of animals injected with 1 mg of testosterone indicated the presence of the spreading factor in only fair amounts.

The administration of 10 mg of anterior pituitary extract alone indicated only fair elaboration or activation of the spreading factor, while 10 mg of anterior pituitary extract plus 1 μg of estradiol benzoate gave a good response.

Discussion. Experiments employing estradiol benzoate indicated that increasing amounts of this hormone produced increasing amounts of the spreading factor, however, even the highest level of estradiol benzoate stimulated little or no spreading factor when injected into adrenalectomized animals. Trentin and Turner(5) found earlier that in the albino rat, mammary gland growth response to estrogen administration depended upon the presence of the adrenal. They postulated that response of the mammary gland to estrogen was due to the ability of this hormone to stimulate the secretion of an adrenal cortical steroid with which it synergized. It was suggested that this steroid was identical with or closely related to progesterone.

It is well known that progesterone is effective in stimulating mammary gland growth. Progesterone was found to be effective also in stimulating the mammary gland spreading factor. The extent of the elaboration or activation of the spreading factor in response to combinations of estradiol benzoate and progesterone showed that optimal synergism was attained when a high level of progesterone and a low level of estradiol benzoate was employed. These observations are similar to those reported upon the stimulus of mammary gland growth by these hormones. In contrast to our inability to obtain production of the spreading factor with estradiol benzoate in adrenalectomized animals, it was found possible to obtain stimulation in adrenalectomized animals when estradiol benzoate and progesterone were administered together, thus confirming the role of the adrenal in mammary gland physiology.

The production of the mammary gland spreading factor by the anterior pituitary extract and the increased response when small amounts of estradiol benzoate were added is consistent with the mammary gland growth produced by anterior pituitary extracts alone and in combination with estradiol benzoate. De Graff, de Jongh and Paesi(6) suggested that testosterone might produce better mammary gland growth than an estrogen-progesterone combination. The close relation between mammary gland growth and production of the spreading factor prompted the trial of testosterone. However, the results obtained with this hormone were distinctly poorer than those obtained with the estradiol benzoate-progesterone combination.

In the previous study(1) the maximum amount of spreading factor was observed in the mammary glands of rats pregnant 12 days. In none of the present experiments did the hormone treatment stimulate the elaboration or activation of an amount of spreading factor equal to the 12th day of pregnancy. However, it will be seen that with certain hormone combinations, the amount of spreading factor extracted equaled that obtained at 10 days of pregnancy. The observations presented in

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this paper suggest that the hormones regulating mammary gland growth are responsible for the elaboration or activation of the spreading factor. Even the amounts of hormone and the hormone combinations which have produced the greatest mammary gland growth were observed to produce the largest amounts of the spreading factor. Thus the amount of spreading factor present and extracted from the glands could well be related to the number of mammary gland cells present.

Summary. 1. Hormones which stimulate mammary gland growth cause elaboration or activation of the mammary gland spreading factor. 2. Increasing amounts of estradiol benzoate caused increasing amounts of the spreading factor to be elaborated or activated in the mammary glands of castrate animals, but little or no spreading factor was found to be present in the mammary gland extracts of estradiol-benzoate treated, castrate-adrenalectomized animals. 3. Increased amounts of progesterone alone caused increased production of the spreading factor, however, the largest amount of progesterone employed did not give as good response as half the level of progesterone plus a little estrogen. 4. Best elaboration or activation of the spreading factor in response to treatment was with a combination of 1 μg estradiol benzoate and 5 mg progesterone. Other combinations of estrogen and progesterone gave graded responses. The castrate-adrenalectomized animals showed as good production of the spreading factor in response to estradiol benzoate-progesterone as castrate animals. 5. An anterior pituitary extract alone produced only fair amounts of the mammary spreading factor. When a low level of estrogen was added much better response was obtained. 6. One mg level of testosterone caused production of fair amounts of the spreading factor. However, the estrogen-progesterone combination gave a better response. 7. Ten days' treatment with some combinations of hormones caused as much elaboration or activation of the mammary gland spreading factor as was extracted from rats pregnant 10 days.

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Localization of a "Feeding Center" in the Hypothalamus of the Rat. (18766)

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Development of obesity in animals following destructive lesions placed in or ventrolateral to the ventromedial nucleus of the hypothalamus is a well-established fact(1-6). This obesity has been shown to arise primarily from a marked increase in food intake, or from what has been termed "hyperphagia."

In an experimental investigation being carried out to discover the effect on food intake, etc. of small electrolytic lesions placed by the Horsley-Clarke instrument in other areas of the hypothalamus of the albino rat, a well-localized area has been found in the lateral hypothalamus, bilateral destruction of which is followed by a complete absence of eating. This area is situated in the extreme lateral portion of the lateral hypothalamus at the same rostro-caudal level (coordinate) as the ventromedial nucleus (Fig. 1). Small, bilateral, electrolytic lesions produced in this area by a current of 1 milliampere used for 15 sec, completely inhibit food intake to the point where the rat dies of starvation. On the other hand, after a unilateral lesion in this area, the animal goes on eating normally; when another

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* Rockefeller Foundation Fellow.

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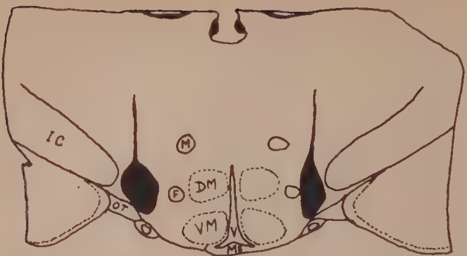

FIG. 1. Section through the rat's brain passing through the hypothalamus in the tuberal region, rostral to the pituitary stalk and caudal to the optic tract. The heavy shaded areas represent bilateral lesions in the lateral hypothalamus, lateral to the fornix and medial to the internal capsule. The dorsal prolongations represent electrode tracks. Abbreviations: D. M., dorsomedial nucleus of the hypothalamus; F., fornix; I. C., internal capsule; M., mamillo-thalamic tract; M. E., median eminence; O. T., optic tract; V., third ventricle; V. M., ventromedial nucleus of the hypothalamus.

unilateral lesion is made in the corresponding area of the opposite side, however, the animal then ceases to eat. This area is so well circumscribed that if the destructive lesion is placed even 0.5 to 1 mm away from it in any direction, this complete absence of eating does not develop. Bilateral lesions made in adjacent areas may result in either decreased eating or failure to eat for the first few days after the operation, but such animals resume eating within a short period.

If the rat is first made hyperphagic and obese by placing bilateral lesions in and around the ventromedial nucleus, and then bilateral lesions are produced in this area of the lateral hypothalamus, the hyperphagia at once yields to non-eating and such an animal also will not eat at all even though it dies of starvation. Unilateral destruction of this area in these hyperphagic animals does not stop the animal from eating but later when a lesion is also made on the other side, it at once abolishes eating. Large electrolytic lesions involving the ventromedial nucleus and spreading into any other part of the hypothalamus, whether anterior, posterior, or lateral, produce hyperphagia and obesity, provided they do not encroach upon this area laterally. If they involve these lateral areas, bilaterally, the animal does not eat.

Experimental work being done on cats has also yielded similar results.

This lateral hypothalamic area, in addition to containing some portion of the cells of the lateral hypothalamic nucleus, has a large collection of fibers passing through it, prominent among which are those from the medial forebrain bundle, stria terminalis, direct amygdalo-hypothalamic fibers, etc.(7). It is premature as yet to say whether the failure of eating is due to destruction of certain cells or some of the fibers; but as lesions produced in the adjacent areas, which might damage identical fibers, do not produce this effect, the probability is more in favor of some cells peculiar to this region. It may be mentioned here that lesions of amygdaloid nuclei in rats have not led to any change in food intake. Also, since destruction of the ventromedial nuclei and their surrounding areas produces hyperphagia and consequent obesity only if this lateral hypothalamic area is intact, the lateral area would appear to play a more important role as regards food intake than the medial hypothalamic structures. It is thus probable that of the two "centers," this lateral mechanism exerts the more basic type of control over food intake, and that its activity is regulated by the medial hypothalamic structures.

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Sublingual Administration of Heparin. (18767)

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There is general agreement on the advantages of heparin as an anticoagulant. However, the methods of administration used to date have not been entirely satisfactory(1,2). It occurred to one of us (J.L.) that some of the disadvantages of these methods, notably the pain on intramuscular injection, the frequency of injection, and the necessity of sterilization, might be avoided by use of the sublingual route. Accordingly, this method was tried and found effective.

Method. Sublingual wafers containing 125 mg of sodium heparin were prepared. The Lee-White 3 tube technic(3) was used for the determination of coagulation time. The pellet of heparin was then placed in the sublingual pouch, where it rapidly disintegrated. Absorption was usually complete in 10 min. The coagulation time was determined prior to the administration and at one-half, one, 2, 3, 4, 6, and 7 hours after the administration of the pellet.

Results. The results obtained are shown in Fig. 1. A therapeutic level is obtained within one-half hr, and maintained for 4 hr.

Discussion. This method makes available a practical means for the easy administration of heparin. In addition, there is no known toxicity for this substance(4) and overdosage is not a problem because of the short duration

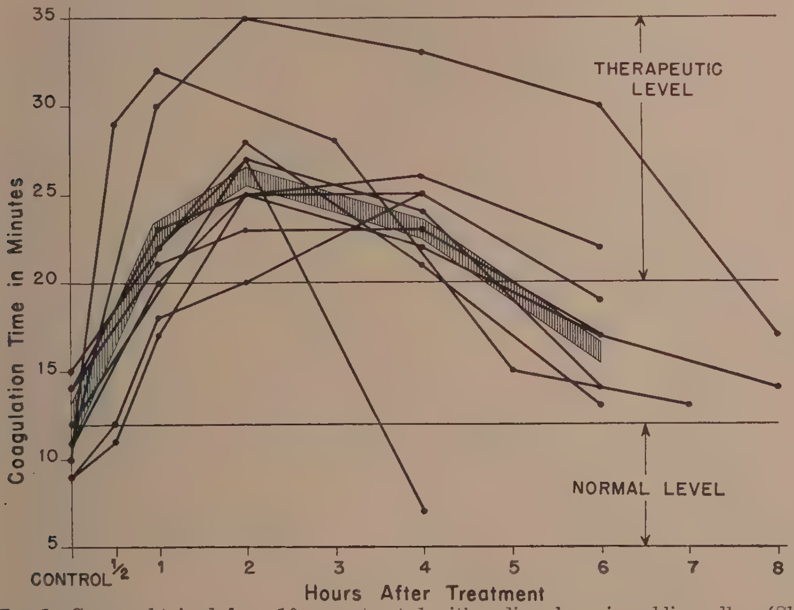

FIG. 1. Curves obtained from 10 cases treated with sodium heparin sublingually. (Shaded area is composite curve of avg.)

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of the anticoagulant effect.

The method fulfills a need expressed by Wright(5) and others for "an anticoagulant which can be taken by mouth and can produce an anticoagulant effect within one hr."

The importance of such a drug in the treatment of frostbite is indicated by the work of Lange(6) and others. The value of initiating such treatment in the field before the hospitalization of a frostbitten soldier is obvious. We believe that by virtue of its rapid effect and ease of administration, the sublingual route may well become the method of choice for the early anticoagulant treatment of myocardial infarction(7), pulmonary embolism, and thrombophlebitis, and in vascular surgery. Further study is indicated to properly assess its place in the anticoagulant armamentarium for the treatment of thrombo-embolic diseases.

Conclusion. Sodium heparin is a practical and effective anticoagulant when administered sublingually. By this method, the coagulation time of the blood was consistently prolonged to the therapeutically effective levels for 4 hr.

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Clinical and Metabolic Study of 17-Hydroxycorticosterone (Kendall Compound F); Comparison with Cortisone.* (18768)

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Additional investigation of Kendall's Compound F was prompted by the probability of its being an important constituent of the adrenal cortex(1,2), its close structural similarity to cortisone, and its effectiveness in rheumatic disorders(3). The present study was undertaken primarily in order to compare the clinical and metabolic effects of 17-hydroxycorticosterone-21-acetate† (Cpd. F) with those produced by cortisone acetate,† employing similar conditions and methods to those previously reported(4).

Case report. A.C., a 38-year-old woman, was admitted to the metabolic ward of the Presbyterian Hospital with active rheumatoid arthritis of 14 years' duration. The diagnosis was made on clinical and laboratory grounds, and was substantiated serologically. After a preliminary period on a constant diet and fluid intake, she received 50 mg of Cpd. F intramuscularly at 12-hour intervals for 24 days. The steroid was then replaced by a placebo (cholesterol suspension†) for one week, following which cortisone was administered intramuscularly at the same intervals and dosage.

Results. (Fig. 1). The administration of both Cpd. F and cortisone was associated with slight insomnia, a moderate degree of increased mental "speed-up" and emotional

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* This study was supported in part by a research grant from the National Heart Institute and Division of Research Grants and Fellowships, U.S.P.H.S., and was aided through the generosity of the Albert and Mary Lasker Foundation, the Albert H. and Jessie D. Wiggin Foundation, and the Masonic Foundation for Medical Research and Human Welfare.

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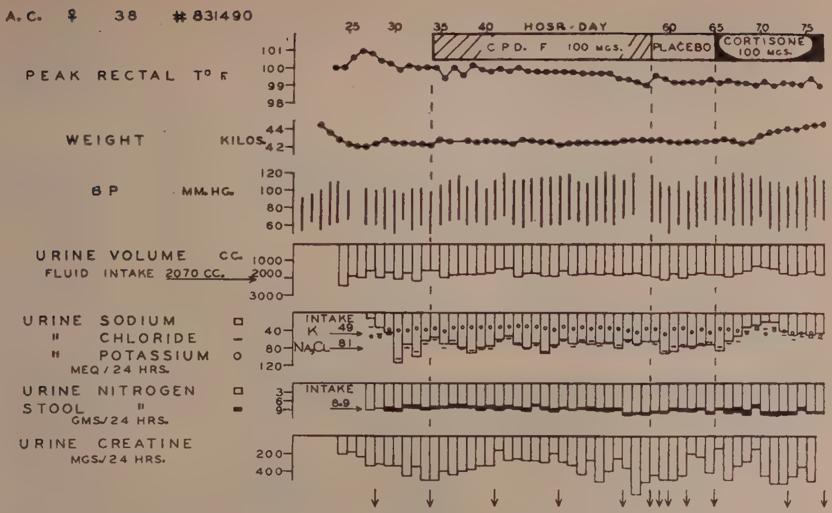

SERUM CO$_2$ CONTENT	MEQ/L.	29.5 28.5 26.2 28.2 27.7 28.7 33.0
" CHLORIDES	"	97.2 97.9 100.2 98.9 99.4 99.0 100.6
" SODIUM	"	134.5 136.1 136.7 136.7 137.1 135.7 134.7 138.1
" POTASSIUM	"	4.2 4.4 4.0 3.8 4.1 4.4 4.2 4.2 3.8
" CALCIUM	MGS/100 cc.	9.7 9.3 9.9 8.7
" INORG. PHOS.	"	3.3 3.3 4.3 3.5 30
" CHOLESTEROL FREE	"	69 73 77 78 80 69 66
" TOTAL	"	233 255 277 287 286 247 239
FASTING BLOOD SUGAR	"	59 120 79 77 94 69 70 75 73
1 1/2 HR p̄c " "	"	182 95 222 185 208 165 196
SERUM PROTEINS		4.0/3.1 3.9/3.0 4.2/3.1 4.2/3.2 4.2/3.3 4.2/2.5 3.8/2.5
" UREA N	MGS/100cc.	10 13 9 11 10 10 12
HEMATOCRIT	% CELLS	33.4 32.6 32.3 34.6 34.4 32.8 32.8 30.1
ERYTH SED RATE	MM/HR.	63 111 103 102 91 59 43 29 44 26 16
17 KETOSTEROIDS	MGS/24 HR. URINE	9.5 8.8 8.3
"CORTICOIDS"	"	1.6 2.3
BMR	%	+1 -6 -3 +1 +6 +3 -1 +2 -5 -2 -10 -10 -6
RADIOACTIVE IODINE UPTAKE	%	43 35
PROTEIN-BOUND IODINE	µG/100 CC.	6.4 5.2 3.5 3.1 1.6
POLYS	CELLS/CU. MM.	3940 3170 2260 3460 3280 4350 5150
LYMPH	"	1690 1160 1700 2800 3060 2050 3070
EOS	"	69 69 331 44 25 38_{10} 25_{10} 75_{30} 19
RETICS	%	0.6 0.7 1.4 0.9 (↑ ACTH) 1.4 1.2
HGB	GMS.	9.7 11.0 10.3 9.9 11.4 9.7 10.2

FIG. 1. Rheumatoid arthritis—treatment with Cpd. F and cortisone.

tension, and an increased appetite. The patient's appearance changed during the Cpd. F period in that her face seemed slightly fuller and more rounded, amenorrhea developed (last menstrual period began on the 20th hospital day), but no further clinical signs or symptoms of hyperadrenocorticalism were evident throughout the study. Repeated electroencephalograms while on Cpd. F showed identical normal patterns, but significant changes developed when subsequently on maintenance cortisone(5). Neither steroid gave rise to alterations in serial teleroentgenograms or electrocardiograms. Both agents produced subjective and objective improvement in the arthritis with disappearance of pain except on motion, increased mobility, reduction in joint swellings and modification of the erythrocyte sedimentation rate. Symptoms returned on the fifth day after stopping Cpd. F, and on the second day after the withdrawal of cortisone. The positive streptococcal agglutination test and the elevated titer of sensitized sheep cell agglutination remained unaffected by either steroid. As judged by weight, evidences of hemodilution, and the

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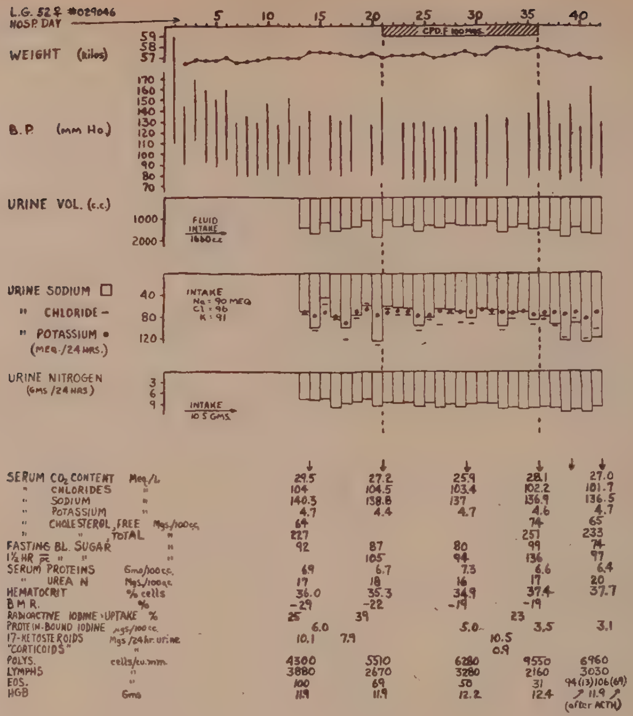

		↓	↓	↓	↓	↓ ↓
SERUM CO₂ CONTENT	Meq./L.	29.5	27.2	25.9	28.1	27.0
" CHLORIDES	"	104	104.5	103.4	102.2	101.7
" SODIUM	"	140.3	138.8	137	136.9	136.5
" POTASSIUM	"	4.7	4.4	4.7	4.6	4.7
" CHOLESTEROL, FREE	Mgs./100cc.	64			74	65
" " , TOTAL	"	227			251	233
FASTING BL. SUGAR	"	92	87	80	99	74
1½ HR p̄c " "	"		105	94	136	97
SERUM PROTEINS	Gms/100cc.	6.9	6.7	7.3	6.6	6.4
" UREA N	Mgs./100cc.	17	18	16	17	20
HEMATOCRIT	% cells	36.0	35.3	34.9	37.4	37.7
B M R.	%	-29	-22	-19	-19	
RADIOACTIVE IODINE UPTAKE	%	25	39		23	
PROTEIN-BOUND IODINE	µgs/100cc.	6.0		5.0	3.5	3.1
17-KETOSTEROIDS	Mgs /24 hr. urine	10.1	7.9		10.5	
"CORTICOIDS"	"				0.9	
POLYS.	cells/cu.mm.	4300	5510	6280	9550	6960
LYMPHS	"	3880	2670	3280	2160	3030
EOS.	"	100	69	50	31	94(13)106(69) ↗ ↗ (after ACTH)
HGB	Gms	11.9	11.9	12.2	12.4	11.9

FIG. 2. Cpd. F in hypertensive vascular disease (L.G.).

output figures, Cpd. F exerted little influence on salt and water balance, whereas cortisone induced definite retention. Cpd. F appeared to be associated with a slightly negative nitrogen balance but no comparison with cortisone could be made because of the short interval between the administration of the steroids.

Additional effects of Cpd. F may be seen in Fig. 1. Noteworthy were the small or negligible alterations in serum electrolytes, the inconclusive changes in blood sugar (although both drugs produced inconstant and slight glycosuria without ketonuria), the rise in serum cholesterol values, and the shifts in circulating white cells. Cpd. F failed to influence thyroid function as estimated by basal metabolic rate, by measurements of radioactive iodine uptake directly over the gland at 2, 4, and 24 hours, and by measurements of urinary excretion of radioactive iodine over the same periods. Nevertheless, a significant decline in protein-bound iodine was observed which might suggest depression of thyroid function. No manifestations of hypoadrenalism appeared after the cessation of Cpd. F.

Additional cases. Fig. 2 illustrates similar effects of Cpd. F, 50 mg twice daily for 15 days, in a 52-year-old woman with uncomplicated hypertensive vascular disease whose arterial tension fell to normal during the baseline period. No subjective changes were noted by this patient other than appetite increase, but a small degree of salt and water retention was evident as illustrated by the slight weight gain and the sodium and chloride diuresis following withdrawal. The blood pressure was unaffected, and other measurements were comparable to the previous study save that glycosuria was not induced.

A third patient, a 33-year-old uncomplicated hypertensive, received Cpd. F, 50 mg twice daily for 10 days, then 75 mg twice daily for an additional 5 days (Fig. 3). This woman developed slight "nervousness," insomnia, and increased appetite during treatment, and with the increased dosage a rise in "resting" blood

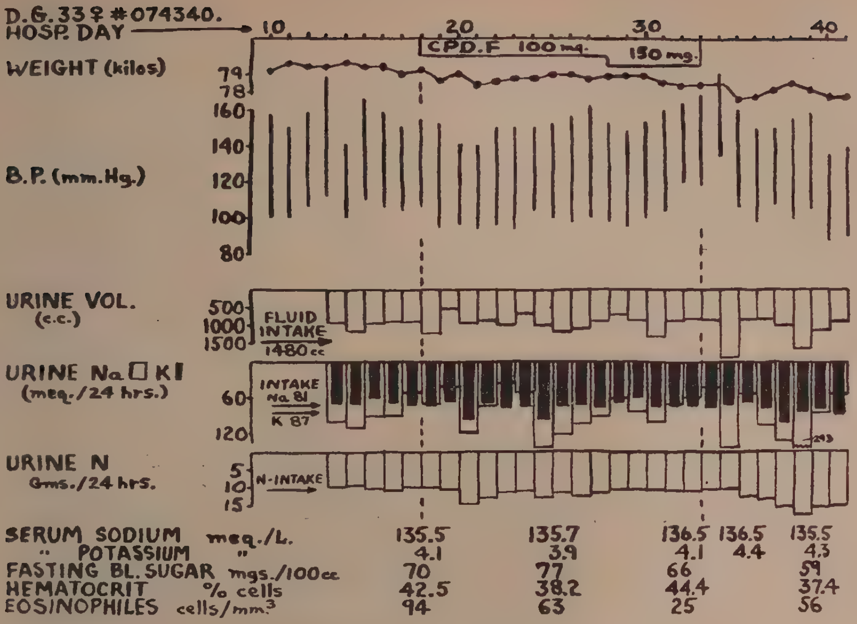

FIG. 3. Cpd. F in hypertensive vascular disease (D.G.).

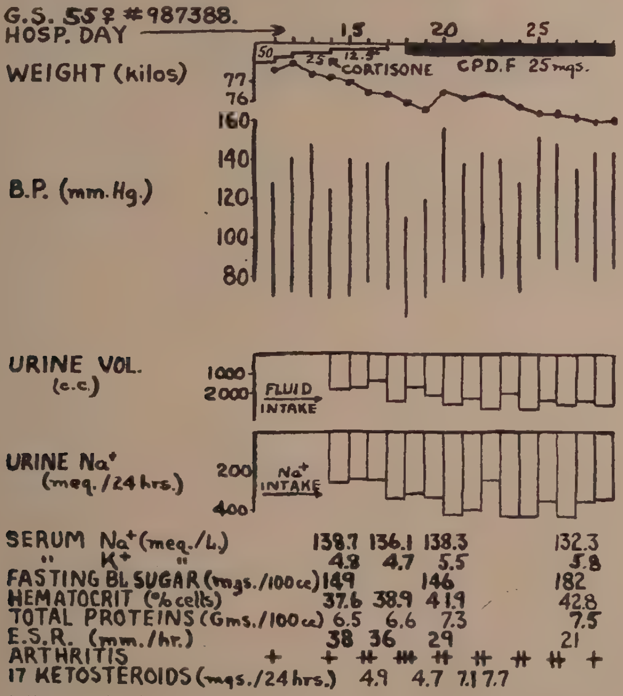

FIG. 4. Cpd. F in an Addisonian patient with arthritis, hypertension and diabetes.

pressure which returned promptly to baseline levels after treatment. Glycosuria appeared throughout the period of steroid administration. On cessation of the injections, there was some water diuresis, a marked urinary loss of sodium, and a rather striking additional loss of nitrogen in the urine.

The final patient, a 55-year-old housewife with hypertensive vascular disease, Addison's disease, diabetes mellitus and rheumatoid arthritis (Fig. 4), received 12.5 mg of Cpd. F daily for 11 days shortly after the gradual dose reduction and discontinuation of cortisone. On the same diet and supplemental sodium chloride as that employed in a previous cortisone study on the same patient(6), Cpd. F administration was associated with a progressive decline in serum sodium, a rise in serum potassium, a negative water and sodium balance, weight loss, some evidence of hemoconcentration, and a slow and inadequate arthritic response. The rise in the 24-hour excretion of 17-ketosteroids during Cpd. F therapy had also been noted previously in this patient after cortisone. On the conclusion of this experiment, a shift to cortisone in the same dosage resulted in weight gain, salt and water retention, restoration of normal serum values, and greater amelioration of the arthritis. Both agents were associated with an increased hyperglycemia and glycosuria.

Discussion. These preliminary studies, representing short-term observations on a small group of patients treated with Cpd. F, suggest again that this steroid exhibits similar effects to those recorded after cortisone(7). However, the comparative data would indicate that Cpd. F is somewhat less active, particularly in the realm of salt and water metabolism. It is of interest that the patient with hypoadrenalism, arthritis and other disorders again showed an anti-arthritic response to small doses, that electrolyte equilibrium could not be maintained on Cpd. F; whereas cortisone restored the balance of electrolytes and seemed to be somewhat more effective as an anti-arthritic agent. The rise in 17-ketosteroid excretion values in this Addisonian subject after Cpd. F and cortisone(6) raises the possibility that certain 11-oxy-steroids, at least in the absence of normal adrenal cortical tissue, are capable of conversion to this end-product. Finally, attention should be called to the divergence of radioactive iodine uptake and the serum level of protein-bound iodine as indices of the effect of Cpd. F on thyroid activity. This aspect is being further investigated (S.C.W.).

Summary. The clinical and metabolic effects of parenterally-administered Cpd. F were investigated and compared with those of cortisone. Preliminary observations suggest similar responses but with Cpd. F appearing to be somewhat less active.

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Electrocardiogram of the Beagle Dog. (18769)

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Information concerning the electrocardiogram of the normal dog(1-4), is scanty, scattered, conflicting and based on relatively few observations in various, usually mixed, breeds. This report describes, in terms of standard

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* This work was done under a grant from the U. S. Public Health Service, Division of Research Grants and Fellowships.

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indices, the electrocardiographic patterns observed in 50 tracings taken on 32 normal beagles, ranging in age from 5 to 28.5 months (average 17.5 months) and in weight from 6.0 to 12.5 kg (average 9.5 kg).

Method. Because of the variability of results obtained in the lateral position(1,2), the supine position, which gave reasonably reproducible tracings, was used exclusively. The dogs, unanesthetized and untrained, were tied to a trough-shaped operating table. Plate electrodes with ample jelly were applied without shaving since preliminary trials had shown that tracings made with and without shaving were identical. A direct-writing machine was employed.

Results. The data are presented in Table IA. The heart rate of the beagle is about twice as rapid as that of man, and the duration of the various components of the cardiac cycle commonly measured is correspondingly shortened. Sinus arhythmia, which was the rule, was the only arhythmia noted. The axis of the QRS complex, measured by the Triaxial Reference System(5) lies farther to the right than in man. Fig. 1, depicting typical tracings in 2 dogs, indicates that the electrocardiogram in the supine position generally resembles that of man in the same position. It sometimes differs, however, in that elevation and depression of the S-T segment up to 1 mm are noted frequently, chiefly in Leads II and III and in aVF. T-I was usually found to be isoelectric; T-II and T-III were always upright. T was always upright in aVF, and usually inverted in aVR and aVL. Marked notching of the T wave was noted in one animal.

The electrocardiographic position of the heart was found to be vertical or semi-vertical, according to the convention of Wilson(6). Two rather distinct patterns were noted; a large group, in which a small or no S wave was present in aVF, associated with QS complexes

TABLE I. Index of Electrocardiographic Standards for the Beagle Dog.

A	Age, mo.	Wt, kg	Rate, min.	P-R, sec.	QRS sec.	QT, sec.	Axis of QRS	Magnitude of deflections in mm*																									
								I				II					III				aVR				aVL				aVF				
								Q	R	S	T	P	Q	R	S	T	Q	R	S	T	Q	R	S	T	Q	R	S	T	Q	R	S	T	
Mean	17.5	9.5	138	.106	.046	.19	72°	0	3.5	0	0	3	0	18	2.5	3	1	15	4	3	12	1	0	−2	6.5	2.5	0	−1.5	0	17	3	3	
Max	28.5	12.5	180	.12	.06	.24	86°	2	7	0	1	4	2	27	10	8	2	25	14	10	21	5	0	−5	15	7	0	−6	2	25	11	8	
Min.	5	6	90	.08	.04	.14	30°	0	1.5	0	−1	1	0	10	0	.5	1	9	0	1	7	0	0	0	2	0	0	0	0	11	0	1	
% occurrence of deflection								12	100	0	32	100	10	100	68	100	6	100	84	100	100	60	0	95	100	71	0	95	9	100	62	100	
B Mean variation of serial tracings			27	0	0	.02	4°	0	1	0	.5	.5	0	2	.5	1.5	0	2	.5	1	3.5	1.5	0	1	3	1.5	0	.5	0	2	.5	1	
Max variation of serial tracings			65	.02	.01	.04	8°	2	1.5	0	1	1	0	6	2	3	1	3	2	2.5	7	3	0	2	9	3	0	2.5	.5	4	4	3	

* Standardization: 1 mv = 1 mm.

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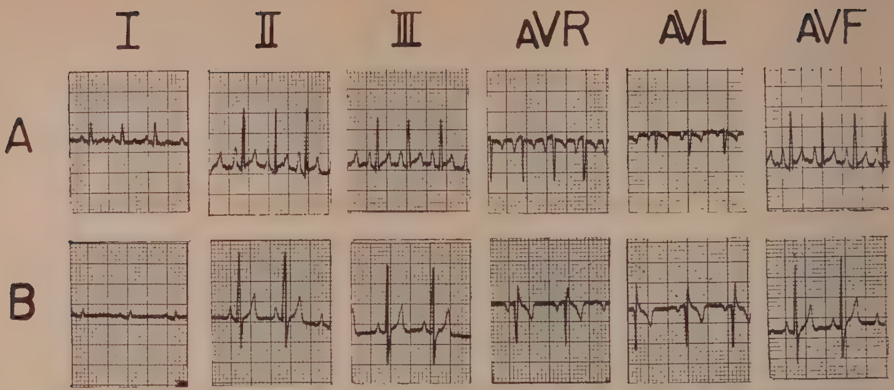

FIG. 1. Electrocardiograms of 2 normal beagle dogs, A 18 months, B 25 months of age representative of the 2 electrocardiographic patterns encountered. QRS complexes retouched.

and inverted T waves in aVR and aVL (Fig. 1A), and a smaller number in which a prominent S wave in aVF was associated with QR complexes and inverted T waves (Fig. 1B) in aVR and aVL.†

Tracings were repeated in the same animal once in 4 instances, twice in 5, and 4 times in one instance. The greatest variation in the serial tracings of any one animal, and the average of these variations, are included in Table IB. The variations encountered are comparable to those described by Mainzer (3) for the sitting position and are much less marked than those previously described for the lateral position. In no instance did the electrocardiographic position or pattern of the heart change in a given animal. In one case notching of the T wave was absent in the first tracing, and present in the subsequent two tracings.

One electrocardiogram was made of a female, unspayed cocker spaniel, aged 6½ months; the findings were in every way comparable with those of the beagles.

Comment. Electrocardiography of the untrained, unanesthetized dog proved to be a simple and practical procedure. There was a varying degree of distortion due to muscular activity but only rarely was it impossible to obtain a satisfactory tracing. The variations in serial tracings were more marked than in man, but not so great as to preclude comparison with one another. In no instance did a positive T wave in Leads II, III and aVF become negative.

It is probable that for the young beagle dog, and possibly for comparable breeds, the data given in Table IA represent a range of values which may be considered as normal, and that findings differing markedly from these may well be indicative of abnormality. Such have been reported by others(8), and have also been encountered by us in studies of chronically diabetic dogs. Further studies as to the influence of breed, size, age and various disease states on the electrocardiogram of the dog would be of value.

Summary. 1. The taking of the electrocardiogram of the untrained, unanesthetized dog is a practical procedure. 2. In the supine position, the variations in serial tracings are small enough to render the results useful. 3. The electrocardiogram of the normal, young beagle dog is described and a table of normal values is presented.

The authors are indebted to Dr. Richard J. Jones for criticism and suggestions.

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† These two groups are of theoretical interest since, according to the convention of Goldberger(7) the first represents forward rotation of apex, and the second backward rotation.

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Biochemical Studies on Blood of Guinea Pigs Treated with Pituitary Adrenocorticotrophic Hormone and Cortisone. (18770)

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In the course of investigating the effect of the pituitary adrenocorticotrophic hormone (ACTH) and cortisone on anaphylaxis in the guinea pig(1), the opportunity presented itself of studying the effect of these hormones on certain of the constituents of guinea pig blood. This was deemed worthwhile because of the paucity of findings on the normal guinea pig and because available findings are seldom grouped together in a single publication. This report presents averages of about 65 determinations taken from apparently healthy animals, also values obtained on the effect of the above hormones on the blood cell picture, serum proteins, cholesterol, chloride, uric acid, creatine, creatinine and glutathione.

Materials and methods. ACTH and cortisone: The preparations of ACTH used in these experiments were Armour Lot 77-S having a potency of 41% of Armour Standard La-1-A and Lot 128-105 with a potency equivalent to 160% of the standard.* The cortisone was used in the form of the acetate marketed by Merck and Co. under the name, "Cortone Acetate." *Antibodies, antigens and sensitization technics*: The antiserum used for passive sensitization was rabbit anti-egg white. The guinea pigs were inoculated by the intravenous (i.v.) injection of 5 ml/kg of this serum. After 2 hr, they were challenged by the i.v. injection of 0.4 ml of a dilute fresh egg white solution (1:8 in physiological saline). Active sensitization to horse serum was accomplished by injecting subcutaneously 1.0 ml of the antigen diluted 1:10 with saline. Challenge was performed on about the 13th day following sensitization by the i.v. injection of 1.0 ml of undiluted antigen.

Histamine shock: Histamine shock was effected by the i.v. injection of histamine, 0.4 mg/kg, as free base. This has been determined(2) as being the MLD_{100} dose. *Blood smear studies*: Cell counts of the mononuclear and polymorphonuclear series were carried out by the routine differential count of a peripheral blood smear stained either by the Wright or Giemsa technic. *Chemical determinations*: Cardiac blood was withdrawn from the guinea pigs for the chemical determinations; the animals in the treated groups were in a state of anaphylactic shock. *Uric acid*: The method of Brown(3) was used as described except that ½ of the original quantities were taken in order to conserve blood serum. *Creatine and creatinine*: The Folin and Wu(4) procedure was utilized for these determinations except that they were carried out on 1/5 the original quantities. *Glutathione*: Determinations were carried out on serum according to the procedure of Benedict and Gottschall(5), as described by Snell and Snell(6), except that it was adapted for the photoelectric colorimeter, the degree of absorption being read at 580 mμ. *Chloride*: This procedure was essentially a modification of that of Snell and Snell(6) in which the chloride determination was adapted as a chromate determination to permit reading in the photoelectric colorimeter. To one ml of a Folin-Wu(4) blood tungstic acid filtrate in a 15 ml centrifuge tube were added about 10 mg of magnesium carbonate to neutralize any possible trace of excess acidity, followed by about 5 mg of dry silver chromate and 9 ml of distilled water. After centrifuging 10-15 min at 1500 RPM 1

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TABLE I. Mean Values for Determinations on Blood of Normal and Hormone Treated Guinea Pigs.

		Experimental groups				
Determination		Normal controls	Untreated anaphylactic shock	Untreated histamine shock	ACTH treated anaphylactic shock	Cortisone treated anaphylactic shock
Glutathione	No. det.	61	15	9	35	10
	mg %	42.6 (19.4–64.2)*	46	62.6	41.4	43.2
	t†		.8	4	.4	.1
Creatine	No. det.	45	15	9	34	10
	mg %	1.14 (.19–2.73)	1.80	1.41	1.60	.87
	t		3	1.1	1.9	1.2
Creatinine	No. det.	65	26	9	49	10
	mg %	1.53 (1.10–2.14)	1.49	1.36	1.61	1.39
	t		.6	1.7	.8	1.6
Uric acid	No. det.	67	26	9	48	10
	mg %	2.53 (1.38–3.88)	3	3.77	4	3.23
	t		2.9	5.4	7.5	3
Uric acid/ creatinine	No. det.	64	26	9	48	10
		1.62	2.02	2.94	3.03	2.38
	t		4.7	8.8	6.7	5.8
Cholesterol	No. det.	48	14	8	49	10
	mg %	41.2 (20 –81.5)	44.3	32	45.5	54.2
	t		.7	1.6	1.3	2.3
Total serum protein	No. det.	57	23	9	49	9
	g %	6.02 (4.5–8)	6.10	6.81	7.29	6.07
	t		.3	2.5	4.2	.2
Albumin/ globulin	No. det.	53	23	9	43	10
		1.73	3.06	2.90	2.63	2.23
	t		3.7	5.6	3.7	2.2
Serum chloride	No. det.		11		14	
	mg %		454 (412–490)		480	
	t				4.7	
Mononuclear leucocytes	No. det.	33		10	47	10
	%	82.6 (34–91)		52.1	56.4	57
	t			6.1	5.8	6.4
Polymorphonuclear leucocytes	No. det.	33		10	47	10
	%	16.1 (9–65)		47.1	42.5	42.7
	t			6.3	5.7	6.7
Eosinophilic leucocytes	No. det.	33		10	47	10
	%	1.3 (0–5)		.8	1.2	.3
	t			.6	.2	1.3

* The values in parentheses indicate the range.

† A value, $t > 3$, indicates significant difference based on 98% confidence limit from normal mean.

ml of the supernatant liquid was removed and placed in a 50 ml volumetric flask. To this were added 40 ml of water, 1 ml of 40% acetic acid, 1 ml of s-diphenylcarbazide reagent(6), and finally water to dilute to 50 ml. The solution was well mixed and read in the photoelectric colorimeter at 545 $m\mu$ 15-20 min after adding the reagents. Macro cuvettes were used and the amounts of chloride were translated from a standard curve. *Serum proteins*: Albumin and globulin were determined by the sodium sulfate salting out procedure of Greenberg(7). *Cholesterol*: The technic of Leiboff (8,9) was carried out essentially as described, except for minor modifications in apparatus and volumes. For the extractions a Folin-Wu blood sugar tube was used with a by-pass connecting the constriction, at about the 6 ml mark, with the upper portion at about the 12.5 ml mark. Blood serum, 0.2 ml, was placed on a small square of chloroform-washed filter paper, allowed to dry, then

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placed in a chloroform-washed 10 x 50 mm Whatman extraction thimble caught and held in place in the sugar tube constriction. About 3.5 ml of chloroform were added and the cholesterol was extracted by refluxing for 30 min. After cooling, chloroform was added to the 4 ml mark, 2 ml of a fresh mixture of 1 part of conc. sulfuric acid and 20 parts of pure, dry acetic anhydride were added, the contents mixed well and the color read in the photoelectric colorimeter at 575 mμ after standing in the dark for exactly 10 min. The cholesterol content was translated from a standard curve. The results of the various determinations are summarized in Table I.

Results and discussion. Histamine shock, passive and active anaphylaxis: Neither ACTH nor cortisone had any effect on the fatal outcome of the reaction, regardless of dosage, number of injections and total dose, time relationship of the challenge dose to the pretreatment, etc. The details are published elsewhere(1).

Chemical and cellular studies: The figure listed as normal for most of the values was the average of a number of determinations taken from apparently healthy guinea pigs. Some values were also obtained from guinea pigs shocked passively and in histamine shock, neither group having had any previous ACTH or cortisone therapy. The statistical evaluation of a significant difference for the results obtained indicates that the difference between the normal and the experimental means varies within at least a 98% confidence limit.

Summary. Normal values for the following constituents of the blood of guinea pigs are presented: blood cell count, serum proteins, cholesterol, chloride, uric acid, creatine, creatinine and glutathione. Physiological alterations were produced by the administration of the adrenocorticotrophic hormone and cortisone as evidenced by an increase in the uric acid/creatinine and albumin/globulin ratios, a decrease in the relative mononuclear and an increase in the polymorphonuclear leucocyte counts.

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Rate of the Enzymatic Breakdown of Digitoxin.* (18771)

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The breakdown of digitoxin by strong acids to form digitoxigenin and digitose has been described by Stoll(1) and others. A similar effect by heart and skeletal muscle was described by Fischer(2). However, in neither of these cases was any attempt made to study the reaction rates of these changes. The development of a polarographic method for the analysis of digitoxin (Hilton)(3), in very small quantities has made it possible to study the breakdown of digitoxin and to determine the reaction rate constants for these reactions in dilute acid and in extracts of heart, liver, kidney, and yeast. In the case of the heart extract attempts were made to purify the essential enzymatic component which was responsible for the reaction.

Methods. The breakdown of digitoxin by 0.85% hydrochloric acid was carried out as follows: 9.0 ml of 0.85% hydrochloric acid were placed in each of six 50 ml Erlenmeyer flasks which were shaken in a constant tem-

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* This work was aided in part by a grant from Eli Lilly Co., Indianapolis, Ind.

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perature bath at 29.7 ± 0.05°C for 30 minutes. After this period of time, 0.100 mg of digitoxin in 50% ethyl alcohol was introduced into each flask. Periodically after the addition of digitoxin a vessel was removed, and the contents were extracted with 25 to 35 ml of petroleum ether. The extract was evaporated to dryness and the residue was analyzed polarographically for its digitoxin content. The breakdown of digitoxin by the extracts of various organs and yeast was carried out in the same manner as the hydrochloric acid reaction. In these studies the extracts used were prepared by mixing 10 g of dried, defatted organs[‡] with 100 ml of phosphate buffer (0.20 molar, pH 7.31) and allowed to stand for 1½ hours. After this time, the mixture was filtered, diluted to 100 ml in a volumetric flask and allowed to stand at 5°C for 2 to 3 hours before using. The organs used in this study were: Heart, kidney, liver, and baker's yeast (Fleischman, dried for 24 hours at room temperature).

Results. Of the 5 substances studied, only acid, heart, and liver showed digitoxin splitting activity. A plot of the digitoxin remaining at given time intervals after the addition of the drug may be seen in Fig. 1. This figure is for acid only, however, the plots of digitoxin splitting activity for heart and liver extracts are very similar. Zero time extractions and controlled samples run on 0.85% saline and phosphate buffer show the complete extractability of the digitoxin added. In saline and phosphate buffer, no breakdown was observed in time intervals up to 240 minutes.

The shape of the time-concentration plot, and the fact that a log concentration-time plot is a straight line indicates that this reaction may be a first order reaction. Reaction rate constants at 29.7°C calculated by the equation $K = \frac{2.303}{t} \log \frac{c_0}{c_t}$ were as follows: 0.0765 g hydrochloric acid 6.85 × 10^{-2}, standard deviation[§] 0.48 × 10^{-2}; active principle in 0.9 g dried, defatted heart 5.15 × 10^{-2}, standard deviation 0.14 × 10^{-2}; active principle 0.9 g dried, defatted liver 5.80 × 10^{-2}, standard deviation 0.40 × 10^{-2}.

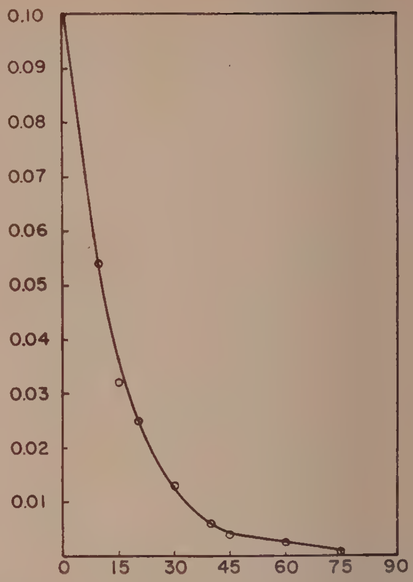

FIG. 1. Breakdown of digitoxin by .85% hydrochloric acid. Ordinates, digitoxin remaining in mg; abscissae, time in min.

A purification of heart extract was attempted by dialysis of the filtered extract against cold running water for 24 hours. This produced a grayish brown precipitate, soluble in phosphate buffer and having approximately 5 times the activity of the residual solution. Further dialysis of the phosphate buffer failed to reprecipitate the active component, but addition of ethyl alcohol in the cold (5°C) to the extent of 50% by volume produced a reddish brown solid which showed a high digitoxin splitting activity. This solid was soluble in phosphate buffer (0.20 Molar pH 7.31) and showed typical protein reactions.

Discussion. The breakdown of digitoxin to digitoxigenin in acid medium has been shown to be a rapid reaction which may be of the first order. The breakdown of digitoxin by heart and liver extracts is similar in type and rapidity. The activity of these tissues appears to be due to some substance present in the tissues and not due to a pH effect. The presence of this substance in heart and liver tissues which splits digitoxin, and thus changes the solubility characteristics, may in some manner explain the action of digitoxin and its mode of excretion.

[‡] The dried defatted organs used in this study were obtained from VioBin Corp., Monticello, Ill. These tissues were dried and defatted at 37° C by azeotrophic process. The medium for both drying and defatting was ethylene dichloride.

[§] Standard deviation of individual determinations from run average.

Summary. A study of the breakdown of digitoxin to form digitoxigenin by hydrochloric acid and extracts of dried heart, liver, kidney, and yeast has been carried out. The results of this study showed the hydrochloric acid and extracts of heart and liver have the ability to cause this breakdown, while extracts of kidney and yeast are inactive in this reaction. Reaction rate studies gave the following rate constants at 29.7°C: 0.85% hydrochloric acid 6.85×10^{-2}, heart extract (10 g dried, defatted powder per 100 ml phosphate buffer) 5.15×10^{-2}, liver extract 5.80×10^{-2}. Purification of the heart extract yielded a reddish brown solid which had a high enzymatic activity and gave typical protein reactions.

The author wishes to express his thanks to Dr. C. L. Gemmill for his advice and guidance in carrying out this work and to Dr. K. K. Chen, of Eli Lilly Co., and Dr. F. E. Kelsey, of the Department of Pharmacology, University of Chicago, who kindly supplied the drugs used in this study.

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Effect of X-Radiation upon Succinoxidase of Rat Kidney. (18772)

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Hammett(1), irradiating a solution of glutathione with radium, found a decrease in sulfhydryl groups and an increase in disulfide groups and suggested that radium irradiation in "non-tissue destroying amounts" had its main effect on cell growth by decreasing the concentration of sulfhydryl. Barron and coworkers(2-4) reported both *in vitro* and *in vivo* experiments showing inhibition of –SH enzymes following small amounts of X-irradiation. In the latter experiments, inhibition was relatively marked in kidney slices from X-irradiated animals with succinate as a substrate, presumably as a result of inhibition of the –SH enzyme, succinic dehydrogenase.

Since succinic dehydrogenase is a widely distributed enzyme whose activity as affected by irradiation could be easily measured, further study of the effect of irradiation on the succinoxidase system seemed indicated.

Methods. Litter-mate Sprague-Dawley rats weighing 150 to 200 g were divided into control and irradiated groups. The irradiated animals received 400 r to 800 r total-body X-radiation (200 kv, 15 ma, and 1.5 mm Cu and 3 mm Bakelite filters). Irradiation was given at approximately 17 r per min, and the animals were killed by a blow on the head within 2 hr following irradiation. Five additional animals were given 800 r (250 kv) at the faster rate of 180 r per min and killed within 7 min after irradiation. The kidneys were removed immediately and 5% homogenates were made in a glass homogenizer with cold distilled water. Kidney homogenates were assayed for succinoxidase, succinic dehydrogenase, and cytochrome oxidase activity by the method of Schneider and Potter(5) as applied by Case and Dickens(6). Kidney

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TABLE I. QO_2 of Kidney Homogenates from Control and Irradiated Animals.

	Succinic oxidase		Succinic dehydrogenase		Cytochrome oxidase	
	No. of animals	Avg $QO_2 \pm$ S.D.	No. of animals	Avg $QO_2 \pm$ S.D.	No. of animals	Avg $QO_2 \pm$ S.D.
Control	19	178.2 ± 18.9	12	68.8 ± 5.4	10	643.3 ± 22.4
After 400 r X-ray	10	191.5 ± 20.9	10	71.3 ± 8.4	7	708 ± 73.6
After 800 r X-ray	5	192.1 ± 12.9	10	69.3 ± 7.6	5	680 ± 7.4

TABLE II. QO_2 of Kidney Slices from Control and Irradiated Animals.

Substrate	None		Succinate		Pyruvate	
	No. of animals	Avg $QO_2 \pm$ S.D.	No. of animals	Avg $QO_2 \pm$ S.D.	No. of animals	Avg $QO_2 \pm$ S.D.
Control	9	21.9 ± 4.3	12	44.7 ± 8.6	10	34.7 ± 3.6
After 400 r X-ray	9	23.8 ± 4.5	11	47.8 ± 7.6	10	38 ± 5.9
After 800 r X-ray	4	22.6 ± 1.7	4	47.8 ± 4.4	3	38.1 ± 4.2

cortex slices were cut freehand. They varied considerably, but all were less than 0.18 mm in thickness. Individual slices were placed in flasks containing Krebs-Ringer phosphate buffer, and oxygen uptake was measured without added substrate and with the addition of 0.3 cc 0.1 M sodium succinate or 0.3 cc 0.1 M lithium pyruvate. Center wells contained 0.2 cc 6 N NaOH. The gas phase was oxygen. After the experiment the slices were removed and dried overnight in an oven at 100°C and weighed. The temperature for all experiments was 38°C and flasks were shaken at 120 oscillations per min. Lithium pyruvate was prepared by the method of Wendel(7).

Results. The determinations of all 3 components of the succinoxidase system in both normal and irradiated animals agree with the figures of Schneider and Potter for rat kidney homogenates. These results are given in Table I. No inhibition of the succinoxidase system occurred even with exposure to 800 r. Five additional animals were exposed to 800 r at the faster rate of 180 r per min and killed within 7 min thereafter. Assay of succinic dehydrogenase, the –SH moiety of succinoxidase, in these animals also showed the same values as the control animals.

The respiratory inhibition in irradiated animals reported by Barron(4) was determined in slices, and not in cell-free homogenates. Similar studies were therefore made on the respiration of kidney slices as shown in Table II. No statistically significant difference could be found between the activities of tissue slices taken from irradiated and from control animals, whether without substrate, or with pyruvate or succinate.

Discussion. The experiments given here were carried out in a manner similar to that employed by Barron *et al.* and as nearly as possible under the conditions which gave these workers their maximum effects. The degree of inhibition of tissue respiration reported by these workers varied considerably, and was not regularly related to the amount of irradiation given or to the time after irradiation at which the tissues were examined. Their animals, the same strain as used in the present study, received radiation ranging between 100 r and 800 r. The tissue slices were examined at times varying, generally, between 4 and 120 hr following irradiation. With the range of doses and within the time intervals just stated, the reported inhibition of succinate oxidation by kidney slices was 36.5% to 79%. The inhibition of the respiration of slices from other organs, using succinate and other substrates, was less marked and less consistent, and this determined the choice of kidney for the present studies. Preliminary experiments with animals killed at 4 and at 24 hr following irradiation also showed no difference in the respiration of the kidney homogenates; therefore, the tissues were examined as soon as possible following irradiation to minimize any possible metabolic reversal of the expected inhibition, and only the higher doses of radiation were given.

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It would appear likely that any additional minor modifications of the conditions used herein would also not cause inhibition of these enzyme reactions. Du Bois and co-workers at the University of Chicago(8) observed no inhibition of succinic dehydrogenase in kidney slices from mice exposed to 2000 r γ rays. Neither did they find, in tissues similarly exposed, any inhibition of the –SH enzyme, adenosinetriphosphatase, which was readily inhibited by irradiation *in vitro*(2). In possible contrast, Dinning and his associates(9) have reported reduction of the endogenous respiration and of the choline and succinate oxidizing activity of bone marrow in 5 mature hens exposed to 300 r of X-rays. Approximately similar decreases were found in all the birds, regardless of the interval, from 1 to 17 days, after irradiation. No claim was made, however, that these reductions represented direct inhibitions of the enzymes by the irradiation. In view of the very rapid changes which occur in bone marrow cells after even moderate irradiation(10), the metabolic changes observed can most probably be ascribed to the altered cellular composition of the tissue.

In contrast to these *in vivo* results, moderate amounts of irradiation have been shown to inhibit –SH enzymes and other enzymes, *in vitro*(2,3,11). But since the addition of proteins and other substances to solutions of enzymes minimizes the inhibition produced by irradiation(11,12), an enzyme can be expected to be more sensitive to irradiation when in a dilute and pure solution than when within a living cell. Forssberg(13), for example, found a 25% inhibition of catalase when it was irradiated with 800 r as a saline solution, but no inhibition of the catalase activity in the rat tissues examined immediately after administration of the same dose to the whole animal.

The emphasis in these studies is on the effects of only moderate doses of irradiation, because of their striking physiological consequences and clinical usefulness, and because of the interest in their possible mode of action. In the rat, hematological changes are caused by much less than 100 r total-body irradiation, and 800 r causes death in 80% or more of the animals. In the present experiments, sulfhydryl enzyme inhibition, not found with doses of 800 r, cannot therefore explain the physiological effects of sub-lethal amounts of irradiation. These results also seem to question any selective *in vivo* effect of moderate doses of irradiation on –SH enzymes. It is probable that following moderate amounts of irradiation the pattern of enzyme reactions in a given tissue may sometimes be changed, but secondarily and as part of the general response, like changes in bone marrow. There appears to be no evidence that the still smaller but still biologically effective doses act *in vivo* directly on any enzyme systems yet known.

Summary. 1. The succinoxidase system, as well as its component enzymes, the –SH enzyme succinic dehydrogenase, and cytochrome oxidase, were determined in rat kidney homogenates within two hours after rats were X-irradiated with 400 r or 800 r. No inhibition of any of the enzymes was found. 2. The metabolism of kidney slices from rats given the above amounts of X-radiation was studied manometrically with sodium succinate and lithium pyruvate substrates. No statistically significant differences were obtained between the oxidative activities of the kidney slices from the control and irradiated animals. 3. The lack of correlation between certain of the *in vivo* and *in vitro* effects of irradiation upon enzyme systems is discussed.

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Response of a Resistant Variant of Leukemic Cells to an Antagonist of Pteroylglutamic Acid. (18773)

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We have reported the development of resistance in 3 separate sublines of a transplantable lymphoid leukemia following serial transplants in mice treated with the MTD of 3 different 4-amino substituted PGA antagonists (1). The variants produced proved to be stable, irreversible heritable changes(2) and recent information(3) indicates that the PGA antagonist has acted as a selective agent, inhibiting the growth of sensitive leukemic cells and allowing the resistant forms, which arise as spontaneous mutants, to survive. The variant forms proved to be resistant to all 4-amino substituted PGA antagonists tested but were inhibited in growth by several other anti-leukemic compounds: 8-Azaguanine, alpha-peltatin(2), triethylene-melamine and X-rays (3). Optimal growth of the variant resistant leukemic cells was obtained when these cells were grown in animals receiving the MTD of PGA antagonists.

The present report concerns the following observations on the behavior of the resistant and dependent leukemic cells of one subline, the AM-resistant subline, resistant to 4-amino-N^{10}-methyl PGA (A-methopterin): (1) the hematologic and histologic response of resistant leukemic cells, as compared with sensitive controls, to various dosage levels of A-methopterin from the MTD level to the LD_{50} level, (2) the effect of Citrovorum factor (C.F.)(4) on the reversal of effects of A-methopterin in the resistant and sensitive sublines and (3) a consideration of the mechanism involved in the adaptation of resistant leukemic cells to dependence upon the PGA antagonist for optimal growth.

Methods and results. Transplant generations 96 to 108 inclusive of the AM-resistant subline and of the corresponding sensitive control subline of acute leukemia L 1210 have been used in the present study. The resistant cells have been carried serially in transplant in strain dba mice receiving 3 mg/kg of A-methopterin* intraperitoneally (.01 cc/g body weight) every other day for 4 days beginning at 48 hours after transfer of leukemic cells. Inoculations of a standard dose of leukemic cells (8×10^5 cells) were done subcutaneously into the right axillary region as described previously(1,2), resulting in a profuse localized growth of lymphomatous tissue which was dissected free of subcutaneous tissues and weighed on a micro-torsion balance. Table I shows the characteristic growth responses of resistant and sensitive leukemic cells to A-methopterin.

The morphology and biologic behavior of the sensitive subline of leukemia L 1210 has been closely observed. Terminally, following subcutaneous transfer of this leukemia, numerous leukemic cells are dispersed in lymph nodes, spleen and liver, enlarging these organs and obscuring the architecture. Leukemic cells appear in the blood a few days after transfer giving a moderately high leukocyte count. By reference to Table II it can be seen that the behavior of the resistant leukemic cells grown in mice receiving 3 mg/kg of A-methopterin is similar to the growth behavior of the sensitive cells grown in antagonist-free mice. There results a florid leukemia with massive lymphomatous growth subcutaneously, a striking secondary anemia, a leukocytosis with escape of lymphoblasts into

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* The folic acid antagonist, A-methopterin, and Citrovorum factor were kindly supplied by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

TABLE I. Comparison of Growth Capacities of Resistant (L 1210 R) and Sensitive (L 1210) Leukemic Cells of Acute Lymphoid Leukemia L 1210 to 4-amino-N^{10}-methyl PGA.

	Transfer generations	No. mice	Dosage (mg/kg)		Mean wt tumor in mg (range)	Dependency ratio
			Daily	Total		
L 1210 R	96–108	78	3	12	833.3 (540.6–1375)	3.92
Resistant		29	0	0	212.7 (143.6–279.9)	
L 1210	96–108	30	3	12	6	—
Sensitive		55	0	0	1190 (983.5–1478)	

the peripheral blood, severe infiltration into subcutaneous and internal lymph nodes, spleen and liver and a mean survival time similar to that observed in untreated mice growing the sensitive leukemic cells. In contrast, note the nearly complete inhibition of leukemia in mice bearing the sensitive subline and given 3 mg/kg of A-methopterin. It was of interest, therefore, to observe the effect of increasing dosage levels up to and including the LD_{50} on the growth response of the resistant leukemic cells. It can be seen that at levels of 5.0 and 7.5 mg/kg (total dose of 20 and 30 mg/kg) profuse localized growth and a generalized leukemia result. At the dosage level of 12.0 mg/kg (total dose 48 mg/kg) which represents the LD_{50} for non-leukemic strain dba mice and mice bearing the sensitive transplant, a reduction of localized growth to approximately 50% has occurred but infiltration into lymph nodes and spleen was still obtained. No deaths attributed to drug toxicity occurred in this group. Thus, it is indicated that the resistant cells now show at least a 50-fold increase in tolerance to A-methopterin, since it requires approximately 0.25 mg/kg (total dose, 1.0 mg/kg) of PGA antagonist to inhibit the localized growth of the sensitive cells to a similar weight level.

In addition to the development of a florid leukemia in A-methopterin-treated mice bearing transfers of resistant leukemic cells, it is apparent that the PGA antagonist supplied to these mice is being used directly as a growth factor by the resistant cells or is being converted for use. It has been found in mice bearing the sensitive leukemic cells and given the folic antagonist at a dosage level of 3 mg or 5 mg/kg every other day for 4 days that various symptoms of vitamin deficiency appear. There results a slight anorexia indicated in a lowered average food intake of 2.0 g per mouse per day and a slight loss in weight over a 7-day period (0.3 g or 0.2%). Untreated mice bearing sensitive leukemic cells during this period gain an average of 2.5 g (12.5%) and consume an average of 2.5 g of food per mouse per day. In contrast, leukemic mice bearing the resistant leukemia and given the PGA antagonist at this dosage level show an average gain in body weight of 2.1 g (10.5%) and consume 2.2 g of food. Other signs of PGA deficiency fail to appear in mice growing the resistant and dependent leukemic cells and supplied with A-methopterin at the 3 mg or 5 mg/kg level. The red pulp of the spleen resembles that seen in the normal animal(5) indicating active hematopoiesis. Numerous megakaryocytes and many foci of nucleated cells of the erythroid series are found throughout the pulp. On the other hand, in mice bearing transplants of the sensitive subline, and given the PGA antagonist, there is definite indication of a deficiency state. The red pulp contains relatively fewer cells with very few megakaryocytes and nucleated red cells. There occurs also a reduction in the number of polymorphonuclear leukocytes. The bone marrow of mice bearing sensitive leukemic cells and given chronic doses of A-methopterin reveals the following signs of PGA deficiency: an increase in cellularity mostly of larger immature cells with many in mitoses and a decrease in megakaryocytes, nucleated red cells and cells of the

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TABLE II. Comparison in Response to 4-amino-N^{10}-methyl PGA of AM-resistant (L 1210 R) and Sensitive (L 1210) Leukemic Cells.*

Transfer line	Dosage (mg/kg) Daily	Dosage (mg/kg) Total	Mean tumor wt, mg	Survival days	Peripheral blood counts Hb	wbc	agran.	gran.	blasts	Organ infiltrations† Lymph nodes	Spleen	Liver
L 1210 R (resistant)	3	12	1362.7	12.4 ± .33	10.1	47760	11940	32299	2821	3+	3+	2+
	5	20	1146		12	24930	7719	16000	1211	3+	3+	2+
	7.5	30	1084		12	46835	9835	34190	2810	2+	2+	2+
	12	48	685.7		13.2	6685	1872	4783	30	2+	1+	—
L 1210 (sensitive)	0	0	1478	12.7 ± .15	11.2	41150	14568	20821	5761	3+	3+	2+
	3	12	0		13.5	11025	6550	4520	55	—	—	—

* Mean tumor wt and histologic data obtained on 10 mice in each group representing 100th transfer of resistant and sensitive cells in dba mice; survival time determined on 20 mice in each group; blood studies represent means of 5 animals in each group.

† 3+ severe, 2+ moderate, 1+ slight, — no infiltration, of leukemic cells into designated organs as determined by microscopic study.

granulocytic series. These changes are evident particularly at the higher dosage level. The picture appears nearly normal, however, in dba mice given the same dosage of antagonist but bearing transplants of the resistant and dependent cells indicating that the PGA antagonist is used, directly or indirectly, in the profuse growth of the resistant leukemic cells.

Citrovorum factor (C.F.) has been shown to be effective in preventing the toxicity of PGA antagonist in *Leuconostoc citrovorum* (6) and in mice (7), and in reversing the anti-leukemic action of these antagonists in mice (8). Since it appears that C.F. on a weight basis, is more effective than PGA in reversing the effects of folic antagonists, it was of interest to study the effects of this factor on the growth of the resistant and sensitive lines of leukemia L 1210 and to determine the blocking effect of C.F. on (a) the anti-leukemic activity of 4-amino-N^{10}-methyl PGA on sensitive leukemic cells and (b) the optimal growth-promoting capacity of this antagonist on resistant leukemic cells.

Citrovorum factor† was injected intraperitoneally in mice at a dosage level of 2 x 10^6 units(4) 24 hours after inoculation subcutaneously of a standard dose of leukemic cells and then 30 minutes prior to injection of 2 mg/kg of 4-amino-N^{10}-methyl PGA. It can be seen from Table III that C.F. did not affect the growth of the resistant leukemic cells, nor was any effect noted on the growth of sensitive leukemic cells. C.F., however, was effective in partially blocking the utilization of the PGA antagonist by the resistant leukemic cells. Only 71% optimal growth of resistant leukemic cells was obtained when C.F. was given prior to 4-amino-N^{10}-methyl PGA. This represents a partial reversal of optimal effect of antagonist equal to 42%. A partial reversal of the anti-leukemic effect of the antagonist

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† The C. F. used in these experiments had an activity for *L. citrovorum* of 3 million units per cc.

TABLE III. Citrovorum Factor (C. F.) and the Effects of 4-amino-N^{10}-methyl PGA on Sensitive and Resistant Leukemic Cells of Leukemia L 1210.*

Experiment	No. mice	Dosage† Daily	Dosage† Total	Relative‡ mean wt	Absolute mean wt, mg (range)	% reversal
			L 1210 resistant			
Amethopterin	42	2	8	1	(758.5–1362.8)	—
Amethopterin + C. F.§	28	2 2×10^6	8 10×10^6	.71 (.59–.87)	(544.1–854.3)	42
C. F.	36	2×10^6	8×10^6	.35 (.31–.42)	(275–580)	—
Controls	36	0	0	.31 (.25–.38)	(195–320.9)	—
			L 1210 sensitive			
Amethopterin	54	2	8	.03 (.0–.09)	(.0–100.8)	—
Amethopterin + C. F.	44	2 2×10^6	8 10×10^6	.28 (.09–.45)	(87–428.3)	29
C. F.	30	2×10^6	8×10^6	.91 (.70–1.12)	(1087–1330.3)	—
Controls	50	0	0	1	(704–1559.4)	—

* Transfer generations 98-102 inclusive. Strain dba mice of both sexes, weighing 18-22 g.

† Dosage of A-methopterin in mg/kg; citrovorum factor dosage in units/kg.

‡ For convenience, optimal growth in both groups considered as 1.0.

§ C. F. inj. intrap. 24 hr prior to first inj. of antagonist, then ½ hr prior to antagonist at 2, 4, 6, and 8 days.

on sensitive leukemic cells was also found (Table III). The reversal of anti-leukemic effect by C.F., as measured by tumor weight, was considerably less than that reported by Burchenal *et al.*(8), who used survival time as a criterion, despite the fact that a considerably higher dosage level of C.F. was used here.

Discussion. It is apparent that we are dealing here with a stable and irreversible, heritable change in leukemic cells such that the requirements for PGA or Citrovorum factor are altered profoundly. Optimal growth of the resistant variants is attained in mice given antagonist at dosage levels far above the MTD, resulting in a florid leukemia with profuse localized growth of lymphomatous tissue, severe infiltration into other organs and leukocytosis with escape of lymphoblasts into the circulation. Evidence that the transformed resistant and dependent leukemic cells are using the PGA antagonist, 4-amino-N^{10}-methyl PGA, either directly or in a converted form is to be found in the lack of signs of vitamin deficiency in mice growing the resistant leukemic cells and provided with antagonist.

The transformation to resistance and dependence has provided a unique and specific modification of a metabolic pathway for biochemical studies. Examples of resistant mutant bacteria requiring specific drugs for growth have been reported(9,10), but it is quite common in bacteria for reversion to occur to the sensitive type. The mechanism of dependence of these transformed leukemic cells on the specific PGA antagonist, 4-amino-N^{10}-methyl PGA or on any of the 4-amino substituted antagonists remains enigmatic. Several theoretical explanations, however, may be considered: (a) the transformed leukemic cells have acquired the ability to convert PGA antagonist to PGA or Citrovorum factor by one of several methods, by deamination of the antagonist at the 4 position of the pteridine ring or by methylation or acetylation of the 4-amino group. This possibility seems unlikely since neither PGA nor C.F. provides for optimal growth of the resistant cells. Furthermore, it has been shown that C.F. actually blocks, partially, the use of the PGA antagonist by the resistant cells. (b) The resistant

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variants have acquired the ability to synthesize their own PGA or C.F. This possibility seems unlikely for the same reasons given in (a) above, (c) the leukemic cells have an increased ability to detoxify the PGA antagonist. From the studies reported here, it is apparent that toxic effects of antagonist do not appear in mice growing the transformed leukemic cells because the antagonist is metabolized in supporting optimal growth. If this were a case of detoxification of antagonist, there would not be expected a differential in the growth capacities of resistant leukemic cells supplied with or lacking antagonist. That is, detoxification could be an explanation for the resistance but not for the dependence characteristic, or (d) the transformed leukemic cells have acquired the ability to use 4-amino-N^{10}-methyl PGA without conversion to PGA or C.F., employing a different mechanism for the synthesis of nucleic acids than that suggested as occurring normally(11,12). This would seem the most likely explanation available at present, although recently Skipper and Burchenal(13) have presented evidence which indicates that the differences in formate fixation in sensitive and resistant strains of the Ak-4 leukemia is only a relative one. It seems improbable, however, from the evidence presented(14), that the resistant Ak-4 cells which were used in their studies are dependent upon PGA antagonist for optimal growth.

Summary. 1. The effect of a PGA antagonist, 4-amino-N^{10}-methyl PGA on resistant leukemic cells of the mouse has been studied. The development of a florid leukemia with profuse localized growth of lymphomatous tissue, leukocytosis with escape of leukemic cells into the blood and extensive infiltration into various organs occurs at dosage levels as high as the LD_{50}. 2. Symptoms of vitamin deficiency did not appear in mice growing the resistant leukemic cells and supplied with a total dose of antagonist as high as 20 mg/kg, indicating that the resistant leukemic cells are metabolizing the antagonist in some manner. Tolerance to 4-amino-N^{10}-methyl PGA has been increased approximately 50-fold. 3. Citrovorum factor did not influence the growth of resistant leukemic cells but partially blocked the optimal growth-promoting effect of the PGA antagonist. 4. Possible mechanisms of action of 4-amino-N^{10}-methyl PGA on resistant and dependent leukemic cells are considered.

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Non-Specificity of Biotin Activity for Leuconostoc.* (18774)

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Literature evidence(1-5) indicates that micro-organisms possess definite configurational specificity with regard to biotin activity.

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It is commonly reported that compounds such as dl-oxybiotin[†] and dl-desthiobiotin possess not more than 50% of the activity of d-biotin, indicating that only the d-forms are effective. Because of our previous demonstration(6) of the unusual nature of biotin activity in *Leuconostoc mesenteroides* and *L. dextranicum,* we investigated the response of these organisms to dl-oxybiotin, dl-desthiobiotin, and to a typical inhibitor of the vitamin, γ-(3,4 ureylenecyclohexyl) butyric acid. Also, since the biosynthesis of aspartic acid is prominent among the numerous functions currently ascribed to biotin(7-10), we studied the effect of biotin and certain of its analogues on the growth of *Leuconostoc* in the presence and absence of added amounts of this amino acid.

Experimental. L. dextranicum elai was the major organism employed. It was carried in sucrose nutrient agar. The composition of the basal medium is given in Table I. It was made up in three-halves strength and distributed in 2 ml amounts in tubes closed with aluminum caps. The remaining components were added in 1 ml volume, and the tubes sterilized 10 minutes at 15 lb pressure. Inocula were prepared by transferring the cultures into 5 ml of the basal medium containing 2% sucrose and incubating 24 hours at 25°C. The cells were separated by centrifugation, washed with saline and resuspended in saline to give a reading of 97% transmission in the Klett-Summerson colorimeter with filter No. 42. On the average this corresponded to a dilution of approximately 1:30. One drop of the washed, diluted inoculum was added to each 3 ml tube of experimental medium. Incubation of experimental tubes was at 25°C. Growth rate was followed by titration with alkali at suitable intervals, bromthymol blue being used as indicator. After correction for the blank titration of uninoculated controls, the results were expressed in terms of ml of 0.01 N acid produced per ml of medium. All measurements were made in triplicate. Assays for aspartic acid content of dried cells were made with *L. mesenteroides* 8042, growth being measured turbidimetrically(11).

TABLE I. Composition of Basal Medium.

Constituent	Conc. per L
Carbohydrate*	25-50 g
L-Glutamic acid	500 mg
DL-Valine	500
DL-Isoleucine	500
L-Leucine	500
DL-Alanine	400
DL-Methionine	400
L-Cysteine	400
DL-Lysine	200
DL-Threonine	200
DL-Phenylalanine	200
DL-Tryptophane	200
DL-Serine	200
Glycine	200
L-Arginine	100
L-Histidine	100
L-Tyrosine	100
L-Proline	100
Adenine	10
Xanthine	10
Guanine	10
Thymine	10
Uracil	10
Nicotinic acid	1
Riboflavin	.5
Thiamin chloride	.5
Calcium pantothenate	.5
Pyridoxal	.2
Pyridoxamine	.2
p-Aminobenzoic acid	.1
Folic acid	.01
NaOAc	25 g
KOAc	5 g
$MgSO_4$	100 mg
NaCl	10
$FeSO_4$	10
$MnSO_4$	10
KH_2PO_4	500
K_2HPO_4	500

* When the carbohydrate used was glucose or fructose, the level was 25 g per l; when sucrose, 50 g per l.

Results and discussion. In Table II are presented results for the effect of d-biotin, dl-

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[†] We wish to thank Dr. K. Hofmann, University of Pittsburgh, for a sample of dl-oxybiotin and Dr. Jackson P. English, Am. Cyanamid Co., Stamford, Conn., for γ-(3,4 ureylenecyclohexyl) butyric acid.

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TABLE II. Effect of Biotin and Various Analogs on Growth of *L. dextranicum* elai in a Synthetic Amino Acid-Fructose Medium Containing No Added Aspartic Acid.

	Acid production in ml .01 N NaOH per ml medium			
Conc. in μg/ml	D-Biotin	DL-Desthiobiotin	DL-Oxybiotin	γ-(3,4-Ureylene-cyclohexyl) butyric acid
0	4.9	4.9	4.9	4.9
1×10^{-6}	6	6	5.4	—
1×10^{-5}	7.4	7.4	8	—
4×10^{-5}	—	—	—	5.4
5×10^{-5}	10	10	10	—
1×10^{-4}	10	10.7	10	—
4×10^{-4}	—	—	—	6.7
1×10^{-3}	10.7	10.1	10.6	—
4×10^{-3}	—	—	—	7.4
2×10^{-2}	—	—	—	9.4
4×10^{-2}	—	—	—	9.4

desthiobiotin, dl-oxybiotin and γ-(3,4 ureylenecyclohexyl) butyric action on the growth of *L. dextranicum* elai in a fructose medium containing no added aspartic acid; each derivative allowed maximal growth to occur. The unusual aspect of the results lies in the fact that dl-oxybiotin and dl-desthiobiotin were found equal to d-biotin in activity, as contrasted with the 50% maximum level of activity reported for these compounds in the case of organisms such as *Lactobacillus arabinosus* and *Saccharomyces cerevisiae*(12-16). Since in other experiments we found dl-desthiobiotin also to be equal in activity to d-biotin for *L. mesenteroides* 683, *L. mesenteroides* 9135, and *L. dextranicum* 8086, it appears that *Leuconostoc* are unique in not showing the configurational specificity demonstrated by other organisms. The fact that the growth curves obtained with the dl-forms showed no increased lag and paralleled that from d-biotin suggests that oxybiotin and desthiobiotin are utilized directly and without conversion to the vitamin. Direct utilization has been established(5,12) for oxybiotin in the case of several micro-organisms. However, evidence has accumulated(13-15) that organisms which utilize desthiobiotin do so only after conversion to biotin. The point is being further investigated for *L. mesenteroides* and *L. dextranicum*, but it appears that the ability to utilize desthiobiotin directly may be more widespread than commonly believed. Lilly and Leonian(16) early demonstrated that desthiobiotin, although it could not substitute for biotin in the metabolism of various lactobacilli, was strongly stimulatory to the organisms if minimal amounts of biotin were present. Thoma and Peterson(17), and Pearlman (18), have reported similar results for various clostridia. The reported functions of biotin are many and diverse, and while for certain organisms the vitamin itself may be required for all these functions, in others it is likely that some of them may be taken over by related compounds. The unexpected finding that a compound reported to be a biotin-blocking agent, γ-(3,4-ureylenecyclohexyl) butyric acid, could stimulate growth in an aspartic acid-free medium further points up the unusual nature of the spectrum of biotin activity in *Leuconostoc*.

The fact that aspartic acid synthesis by *L. dextranicum* elai actually occurred was proven in the cases of media containing d-biotin, dl-desthiobiotin or Tween 80. Tween 80, when used, was present in a concentration of 10 mg per ml. Cells grown under these various conditions were harvested, hydrolyzed, and assayed for their aspartic acid content by accepted methods(11,19). The assay organism used was *L. mesenteroides* 8042,[‡] a strain which cannot synthesize(11) aspartic

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TABLE III. Effect of Biotin, Tween 80, Pyridoxal and Pyridoxamine on Growth of *L. dextranicum* elai in Carbohydrate Media with and without Aspartic Acid.

Additives	Acid production ml .01 N NaOH per ml medium Sucrose No A*	 Sucrose Added A	 Fructose No A	 Fructose Added A
0	7.8	10	2.4	3.5
Pyridoxal, pyridoxamine	12.5	12.5	4	4.2
Biotin	7.8	10	2.8	5.2
Pyridoxal, pyridoxamine, biotin	12.5	12.5	8.3	8.8
Tween	7.8	10.7	3.2	5.5
Pyridoxal, pyridoxamine, Tween 80	12.2	12.2	8.3	8.7

* A = Aspartate.

acid under the influence of either biotin or Tween 80. It was found that the aspartic acid content of the dried cells was essentially the same regardless of whether they were grown in media containing d-biotin, biotin plus aspartic acid, dl-desthiobiotin, or Tween 80, the values obtained ranging from 4.4 to 5%. It seems safe to assume that the amino acid was also formed in the media in which dl-oxybiotin or γ-(3,4-ureylenecyclohexyl) butyric acid was employed.

Literature evidence(9,10,21) indicates that microorganisms which synthesize aspartic acid under the influence of biotin do so via the beta carboxylation of pyruvic acid to yield oxalacetic acid, pyridoxamine then being required to complete the formation of the amino acid. It appeared to be of interest to determine whether aspartic acid synthesis by *L. dextranicum* elai also required the presence of pyridoxamine. Demonstration of such a requirement was rendered difficult by the known (22) ability of *Leuconostoc* to synthesize the B_6 group, although at a rate insufficient to meet the full needs of the organisms. For *L. dextranicum* elai, pyridoxal and pyridoxamine have been shown(23) to be essential only under special conditions. The results obtained are presented in Table III, from which it is seen that in the sucrose medium, addition of pyridoxal and pyridoxamine allowed maximal growth in the aspartic-free medium. Biotin and Tween 80 were without effect. In the sucrose medium containing added aspartic acid, nearly full growth resulted in the absence of pyridoxal and pyridoxamine, although a slight requirement for the B_6 group was still evident. In the fructose medium pyridoxal and pyridoxamine, plus either biotin or Tween 80, were required for full growth in either the presence or absence of added aspartic acid. However, a higher level of growth was observed in the absence of pyridoxal and pyridoxamine when added aspartate was present, again suggesting that synthesis of the amino acid proceeded with oxalacetic acid as an intermediate.

Many investigators have reported(24,27) that oleic acid, or certain of its derivatives, can satisfy the biotin requirement of various organisms only when aspartic acid is furnished in the medium, thus suggesting that oleic acid cannot replace the vitamin in the synthesis of this amino acid. Recently Broquist and Snell (19) found that *Lactobacillus fermenti* and *Clostridium butyricum* represented exceptions to these findings, since the organisms were able to synthesize aspartic acid under the influence of oleic acid. The present study constitutes another case in which the presence of either biotin or an oleic acid derivative allows growth of a microorganism in an aspartate-free medium. However, the results obtained do not offer evidence for the direct participation of biotin or Tween 80 in aspartic acid synthesis by *Leuconostoc*.

Summary. It has been found that *L. dextranicum* elai is unique in that the dl-forms of oxybiotin and desthiobiotin are equal in activity to d-biotin in allowing maximum growth

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in an aspartate-free fructose medium. Since no increased lag period was observed the results suggest that these biotin analogs are utilized directly and without prior conversion to the vitamin. It was also found that γ-(3,4-ureylenecyclohexyl) butyric acid was not a blocking agent for this organism, but instead possessed biotin activity.

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Radioactive Sodium Clearance as a Test of Circulatory Efficiency of Tubed Pedicles and Flaps.* (18775)

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It has been the objective of earlier studies (1-6) on the development of circulation in tubed pedicles and flaps to achieve an accurate test of efficiency of circulation and to encourage its substitution for the current practice of clinical decision as to the interval of time between operative stages in the transplantation of soft tissues. It is apparent that an accurate and dependable test, by shortening the waiting periods between operative stages, would shorten the time of hospitalization and would prevent the complication of tissue necrosis in those cases in which there is uncertainty as to the viability of the pedicle. It is of interest that the clinical problem in cases of peripheral vascular disease is not unlike that presented in the management of tubed pedicles and flaps whose circulation has been artificially contrived. In the former, the clinician seeks to determine the degree of circulatory impairment and to measure the efficiency of the collateral circulation; in the latter, the surgeon is concerned with determination of the earliest date upon which an artificially contrived soft tissue circulation achieves the normal physiological level. Recently, Kety(7,8) showed that the

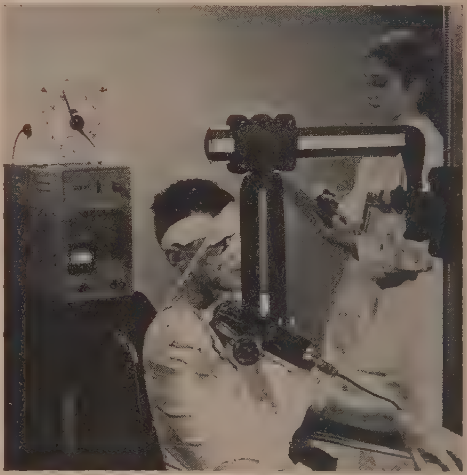

FIG. 1. Mica window of Geiger-Mueller counter placed over end of thoracoacromial pedicle to be transplanted for reconstruction of the nose, upper lip, and chin. Efficiency of circulation of tubed pedicle is determined by comparing clearance of radioactive sodium injected intradermally with that of control area. Relative clearance is determined by comparing half-life of radiosodium in the two areas. If clearance is as rapid from the end of pedicle to be transplanted as it is from the control area, the pedicle should withstand surgical transplantation successfully.

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* Reviewed in the Veterans Administration and published with approval of the Chief Medical Director. Statements and conclusions published by authors are result of their study, and do not necessarily reflect the opinion or policy of the Veterans Administration.

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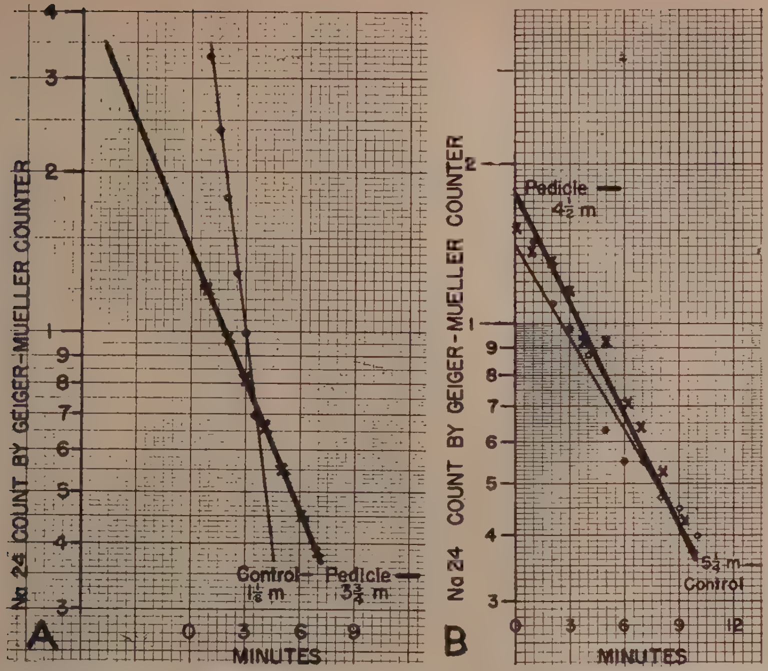

FIG. 2. Clearance of radiosodium from tissue of a pedicle is determined as the net counting rate after subtraction of background rate has been made. This is plotted as a function of time on semi-logarithmic paper. The half-life for resorption, or the time taken for net activity of radiosodium to be reduced to ½ of its original value, is compared with that of control area. In (a) the clearance of radiosodium in control area is more rapid. Circulation in this control area of normal skin is assured to be superior to that in the pedicle. However, in (b) the reverse is true, the clearance of radiosodium as determined by its half-life, being more rapid in the pedicle than in control. The pedicle was transplanted successfully one day following this test.

rate of disappearance of radioactive sodium (Na24) from muscle tissue offers a superior type of assay of the status of the regional circulation in extremities. This observation was supported by the studies of Elkin, *et al.* (9) and of Stone and Miller(10) who showed that lymphatic drainage does not play a significant role in the intramuscular clearance of Na24 and that the radioactive material does not diffuse along intramuscular planes.

It occurred to us that a radiosodium clearance technique might well be adapted to problems of circulation in pedicles of skin and subcutaneous tissues. It is the objective of this presentation to record observations on the clearance of radiosodium as a test of circulatory efficiency in tubed pedicles and flaps as they have been employed in plastic surgical reconstructions.

Technique. An injection of 0.05 cc of isotonic saline solution containing 0.1 to 0.5 microcuries of radioactive sodium (Na24) is made intradermally at a point near the end of the pedicle which is to be transplanted. A similar injection is made as a control in the skin of the same region on the opposite side of the body. Patients are tested under approximately standard conditions of room temperature, test areas and time of day. Na24 has a physical half life of 14.7 hours decaying

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TABLE I. Radioactive Sodium Wheal.

Case	Type pedicle	Location	Defect	Length /width ratio	After construction, days	Min. required for half of initial dose of Na24 to be resorbed		Interpretation	Progress of case
1	Tubed P*	thoraco-acromial, right	symphysis mentis, chin	3:1	79	P	7	2nd move made 5 days after ¾ severance	33 days after transplantation of tubed P to chin. 2 days following ¾ severance of tubed P and 5 days before second end of tubed P transplanted to chin.
						control	3.75		
2	Open P	temporal, left	loss of eye, cheek, and half of nose	2:1	3	P	3.5	test indicated directly P could not be moved	1 day later P elevated, cyanotic, returned to its bed.
						control	1.5		
					48	P	1.75	correct prognosis by test	P successfully transplanted 1 day later.
3	Tubed P	thoraco-acromial, right	nose, palate	3:1	1	P	10.5	P probably would have withstood transplantation at 9 days; correct prognosis by test	tubed P completely detached successfully.
					3	P	4.6		
					5	P	7		
					7	P	5.25		
					9	P	5.5		
						control	4.5		
					12	P	4.75		
4	Tubed P with open P pancake	thoraco-acromial, left	left nasal, malar, upper lip	3:1	4	P	2.25	test indicated base could be elevated	pancake doubly grafted with split-thickness skin graft, to under surface.
						control	3.125		
					39	P	2.75	correct prognosis by test	transplanted successfully 3 days after test.
					66	P	3		second end transplanted successfully 1 day later.
5	Open P	temporal, right	right orbit, malar nasal	2:1	15	P	2.25	"	transplanted successfully 9 days after test.
						control	1.5		
6	Open P	cross leg flap	FCC left tibia, fistula	1:1	19	P	7	"	successfully transplanted 1 day after test.
						control	6	"	
7	Open P	cross thigh, left	right distal leg	2:1	10	P	6.5	"	P transplanted successfully 2 days later.
						control	6.5	"	
8	Tubed P	thoraco-acromial, right	right cheek	3:1		P	5	"	P transplanted successfully 1 day later.
						control	5	"	

* P = Pedicle.

by the emission of high energy gamma rays and beta particles, the maximum range of which in tissue is about 6 mm. A Geiger counter with thin mica window is placed in a fixed position over the site of intradermal injection. The counting rate is measured as a function of time. Since the injection is superficial, the counter will detect the radiation of beta particles and, with lesser efficiency, the gamma rays from the injected radiosodium, thus making it possible to use less than a tenth of the dose employed by Kety(7,8). Some of the radiosodium may be deposited accidentally on the skin and hence will not be cleared. This will give a background counting rate after the resorption of Na24 is complete. The net counting rate is plotted as a function of time on semi-logarithmic paper. Within the limits of experimental error, the plotted points lie along a straight line. From the graph is determined the half-life for resorption, which is the time taken for the net activity to be reduced to one half its original value.

Results. It has been found from the study of nine pedicled flaps in eight patients that, if the rate of disappearance of radiosodium from the tubed pedicle is approximately equal to or more rapid than that of the control area, division and migration of the pedicle can be carried out successfully. In the study of one tubed pedicle, the time required for resorption of the dose of radiosodium to its half-life was more than twice that of the control area. In this case, in an attempt to establish the validity of the test, the pedicle was divided by incision. The degree of cyanosis which developed after division of the pedicle was such that its transplantation, a step which adds further burden to a feeble circulation, could not have been done with good surgical judgment at that time. The rotation and transplantation of a pedicle which shows gross signs of circulatory inadequacy is an invitation for the development of necrosis of the flaps, a complication which defeats the purpose of the reconstructive operation. In this particular case, the cyanotic pedicle was returned to its former bed to await the development of sufficient collateral circulation. A refinement of those circulation tests which depend upon the measure of the rate of resorption of an injection is brought out by the employment of a tourniquet at that end of the pedicle which is to be transplanted. For tubed pedicles a rubber catheter serves as a tourniquet. For broad-based flaps, one or two rubber-shod gastric clamps may be used. In experiences with the saline wheal test, the fluorescein test, the atropine test and the histamine wheal test this technique has been found to provide more complete information since the resorption of the drug depends upon a contrived circulation the afferent and efferent pathways of which must travel the entire length of the pedicle. However, a tourniqueted pedicle clears radiosodium so slowly that the use of this modification of the test is not practical.

In comparing the radiosodium test for the evaluation of circulatory efficiency with other tests such as the saline wheal test, visualization by fluorescein dye and the histamine wheal test, the authors feel that the end point of the radiosodium clearance test is the most accurate of all. It is recorded accurately and by objective measures in a relatively short period of time.

Summary and conclusions. The rate of clearance of an intradermal wheal of radioactive sodium (Na24) has been successfully employed in eight patients as a clinical test for the evaluation of the circulation in tubed pedicles and flaps. The experiences recorded indicate that the radioactive sodium clearance test, because of its simplicity and accuracy, has a wide field of application in the study of problems in the transplantation of soft tissues.

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Alteration of *In Vivo* Propulsive Intestinal Motility by Antihistaminic Drugs.* (18776)

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Studies on the effect of antihistaminic drugs on *in vivo* intestinal motility have made use of the following experimental preparations: Thiry-Vella loops(1), exteriorized loops of intestine(2) as described by Biebl(3) and by Douglas and Mann(4), intestines made "permanently opaque" to X-ray(5), and intestines left *in situ* after laparotomy(6-8). An indirect approach was used by Erspamer and Paolini(9) who reported an effect of antihistaminic drugs on the rate of action of purgatives. Observations of propulsive intestinal motility, as such, have been made by measuring the rate of transport of a charcoal-acacia mixture through the gastrointestinal tract (10). Comparable investigations have been made in which carmine was used as a "marker" for the time periods between ingestion of a meal and defecation of the residue(11).

Most of the technics used to determine the effect of antihistaminic drugs on intestinal motility involve extensive preparative surgery in order to get an *in vivo* preparation which is both easily duplicatable and relatively normal physiologically. In order to avoid this preparative surgery, an attempt was made to investigate *in vivo*, the effect of antihistaminic drugs on only the propulsive aspect of intestinal motility, this being measured by observing the time from ingestion of carmine to its defecation. The studies, here presented, show the effect obtained with the use of the following 5 drugs: Diphenhydramine hydrochloride (Benadryl), tripelennamine hydrochloride (Pyribenzamine), thenylpyramine hydrochloride (Histadyl), phenazoline hydrochloride (Antistine), and phenindamine acid tartrate (Thephorin).

Experimental. Seventy-one male rats of 80-110 days of age were used, that had been obtained from the inbred Wistar stock colony of the Department of Biochemistry. All rats were put into individual wire nutrition cages and fasted for 24 hours prior to the feeding of the dye. The antihistaminic drug was administered by subcutaneous injections of equal size at 9 hours, 5 hours, and 30 minutes, respectively, prior to the time of feeding the dye. All drugs were given in terms of millimoles (mM) per kilo body weight per injection in slightly acid isotonic saline, the solutions having been made up fresh daily from the crystalline salt. The dye was fed as a dry colored checker (Purina Laboratory Chow) which had been prepared by soaking the checkers in an alkaline aqueous solution of carmine followed by room-temperature drying. Each rat was given 2 dyed checkers at the beginning of the feeding period and this

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* The authors wish to express their appreciation to the following for making certain drugs available: Eli Lilly & Co., Histadyl Hydrochloride; Hoffmann-LaRoche, Thephorin; Parke, Davis & Co., Benadryl Hydrochloride; and Ciba Pharmaceutical Products, Pyribenzamine Hydrochloride and Antistine Hydrochloride.

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TABLE I. Effect of Antihistaminic Drugs on Time from Ingestion of Carmine to its Defecation.

Date of exp.	No. of rats	Grp.	Drug	Dose, mM/kg per inj.	Time,* hr	Significance	
						Groups compared	''P''†
11-24	7	I	Saline	—	4.05 ± .45		
11-24	7	II	Benadryl	.16	5.25 ± 1.03	I,11-24::II,11-24	.01
12-11	5‡		No inj.	—	3.80 ± .48	II,11-24::II,12-11	.01
	7		(No inj.)	—	(3.15 ± 1.18)	(II,11-24::II,12-11)	(<.01)
-20	6§		Benadryl	.08	4.65 ± .60	II,12-11::II,12-20	<.05; >.02
11-26	7	III	Benadryl	.04	3.35 ± .67	I,11-24::III,11-26	.06
12-4	7		No inj.	—	4.20 ± .78	III,11-26::III,12-4	.05
-7	7		Benadryl	.12	4.30 ± .95	III,12-4::III,12-7	<.9 ; >.8
11-24	7	IV	Thephorin	.16	''too toxic to eat''		
12-11	7		No inj.	—	4.55 ± .77		
-20	7		Thephorin	.08	5.55 ± 1.08	IV,12-11::IV,12-20	<.1 ; >.05
11-26	7	V	Thephorin	.04	2.45 ± .70	I,11-24::V,11-26	<.01
12-4	7		No inj.	—	3.95 ± 1.04	V,11-26::V,12-4	<.01
-7	4‖		Thephorin	.12	3.80 ± .24	V,12-4::V,12-7	<.8 ; >.7
11-25	7	VI	Histadyl	.12	''too toxic to eat''		
12-11	7		No inj.	—	4.85 ± .70		
-20	7		Histadyl	.04	5.30 ± 1.06	VI,12-11::VI,12-20	<.4 ; >.3
11-25	7	VII	Antistine	.12	5.15 ± .69	I,11-24::VII,11-25	<.01
12-4	7		No inj.	—	4.65 ± .59	VII,11-25::VII,12-4	<.2 ; >.1
-7	7		Antistine	.04	4.20 ± .51	VII,12-4::VII,12-7	<.2 ; >.1
11-25	7	VIII	Pyribenzamine	.12	''too toxic to eat''		
12-11	5¶		No inj.	—	4.65 ± .29		
-20	5¶		Pyribenzamine	.04	4.15 ± 1.45	VIII,12-11::VIII,12-20	<.5 ; >.4
12-22	15	IX	No inj.	—	3.70 ± .55	I,11-24::IX,12-22	<.2 ; >.1
-26	15		'' ''	—	4.05 ± .74	IX,12-22::IX,12-26	<.2 ; >.1
-29	15		'' ''	—	3.60 ± .69	IX,12-22::IX,12-29	<1.0; >.9

* Means and std dev.

† ''P'' calculated by the t test of unpaired variates(12).

‡ Two rats were apparently still showing effects from previous Benadryl administration and not included in these values, but included in values in parentheses directly below.

§ Only 6 rats remaining in this group because one was sacrificed for reasons unrelated to exp.

‖ Only 4 rats in this exp. Remaining 3 were apparently ''too toxic to eat.''

¶ Only 5 rats remaining in this group because remaining 2 died of toxicity following first pyribenzamine administration.

amount of food was supplemented with undyed checkers after the first had been eaten. For all experiments, the injections were given during the daylight hours and the dye was fed at about 7:30 P.M. With such a schedule there were practically no environmental disturbances for the rats during the eating period and they could maintain their normal nocturnal eating habits. The room in which the rats were being observed was kept relatively dark after feeding of the dye. A clean paper towel was placed under each cage one hour after the initial feeding. Thereafter, inspections for carmine-colored feces were made at 15-minute intervals by using a small flashlight, so as to cause only a minimal unregulated environmental disturbance. Inasmuch as some of the drugs caused drowsiness and because an eating animal was essential for this test, the wire on each cage was scratched once, at the time of feces inspection, with a metal spatula in order to give a regulated awakening stimulus.

Results and discussion. The results are summarized in Table I. Benadryl in the dose of 0.16 mM produced an average *increase* in time of 1.20 hours (30%; $P = 0.01$) between the time of ingestion of carmine and its appearance in the feces. In a dose of 0.04 mM, Benadryl caused an average *decrease* in time of 0.7 hour (17%; $P = 0.06$). This reversal in type of activity by Benadryl, as produced by increased dosage, is comparable to that reported by Scudi, Reinhard, and Dreyer(7), who observed the intestine *in situ* and found thonzylamine hydrochloride

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(Neohetramine) to act as a stimulant in small doses and as an inhibitor in large doses. Similar effects have been observed(8) with prophenpyridamine (Inhiston).

The rats which received Thephorin in the dose of 0.16 mM failed to eat and seemed to show signs of toxicity after the last injection. However, the rats which received 0.04 mM of Thephorin showed an average *decrease* in time from ingestion of carmine to its defecation of 1.60 hours (40%; $P = < 0.01$). This stimulation of motility is comparable to but somewhat greater than that seen after 0.04 mM of Benadryl.

Like the rats receiving 0.16 mM of Thephorin, the rats receiving Histadyl and Pyribenzamine in a dose of 0.12 mM, also failed to eat and were apparently poisoned by the drugs. Two of the 7 rats receiving 0.12 mM of Pyribenzamine died in less than 24 hours after the last injection. A dose of 0.04 mM of either Histadyl or Pyribenzamine failed to produce a significant effect.

Antistine when given in a dose of 0.12 mM produced an average *increase* in time of 1.05 hours (26%; $P = < 0.01$). The Antistine, therefore, when given as 0.12 mM showed an inhibition of motility comparable to that seen when Benadryl was given in the larger dose of 0.16 mM. This comparison of Antistine and Benadryl is in agreement with the work of Erspamer and Paolini(9) who found Antistine to be a greater inhibitor of purgatives than Benadryl.

Inasmuch as the rat is relatively resistant to histamine(8) high doses of the antihistaminic drugs, approaching toxicity, were used in order to show the alterations in *in vivo* propulsive intestinal motilities as measured by observing the time from ingestion of carmine to its appearance in the feces. A comparable study in other species has not yet been made. However, both the data of this report and that of other work to which reference has been made, indicate that such a study should be made in order to see whether size of dose and chemical type of drug(13) should be taken into consideration when prescribing antihistaminic compounds.

Summary. 1. Antihistaminic drugs can affect the rate of *in vivo* propulsive intestinal motility as measured by observing the time between ingestion of carmine and its appearance in the feces. In comparable small doses Thephorin and Benadryl stimulate intestinal motility. 2. In larger doses, Antistine and Benadryl produce inhibition. This then shows a reversal in effect by Benadryl dependent on size of dose.

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Isolation of Coxsackie Viruses. Litter Differences Among Suckling Mice.* (18777)

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In planning a large scale study designed to throw light on the role of Coxsackie or C viruses in human disease by determining the frequency with which these agents may be demonstrated in specimens from various sources, an effort has been made to establish rigid technical criteria. It is common practice(1-4) to use one litter of suckling mice for each specimen being tested, although some investigators use two(5), and still others(6)

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* Aided by a grant from the National Foundation for Infantile Paralysis.

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select the mice at random, the animals being pooled and redistributed. Animals are usually observed for a period of 2 to 3 weeks. To limit the possibility of spontaneous or accidental infections of mice, it has been suggested that animal inoculations be limited to one "blind" passage in suckling mice(7). In this laboratory each specimen to be tested for C virus is inoculated into 12 to 20 2-day-old suckling white Swiss mice (CF No. 1) representing a minimum of 2 litters. Animals are observed frequently for a period of 2 weeks. Since, through cannibalism, a large number of animals are devoured, it was decided that at least 6 mice representing 2 litters must survive the observation period of 2 weeks, before a specimen is considered negative on first passage. When no symptoms are observed during the first week following the inoculation, 2 mice from each litter are sacrificed for a "blind" passage during which the above procedure is repeated. Since the possibility exists that adult female mice, although insusceptible to Coxsackie virus infection, might become immune as a result of laboratory exposure and transmit this immunity to their offspring, all normal stock mice are maintained in isolated quarters.

Using the procedure described above, 23 strains of Coxsackie virus have been isolated. Apparent litter differences in susceptibility of suckling mice to these agents on primary isolation were evident in a number of instances. The results with 3 strains recovered from stool specimens are summarized in Table I. Each of 8 litter mates inoculated with specimen No. 16 survived the observation period of 2 weeks, while each of 5 mice in a second litter developed symptoms consisting of tremors of the extremities, ataxia, weakness, cyanosis, and retardation of growth and were sacrificed on the fifth and sixth days after inoculation for further passage in suckling mice. Only 4 of 18 mice inoculated with specimen No. 186 developed signs and symptoms of Coxsackie virus infection. That these 4 mice were members of one of the 3 litters inoculated with this specimen may, of course, have been due to chance. Litter difference in susceptibility of suckling mice to C virus infection on primary isolation is also evident in the case of specimen No. 9. None of 8 litter mates showed signs and symptoms during the observation period, while 4 out of 7 mice in a second litter showed characteristic symptoms. Similar litter differences were observed with the viruses recovered from 5 additional specimens. On second passage in mice no litter difference was observed in susceptibility of animals to strains No. 9 and No. 186. Strain No. 16, on the other hand, was difficult to adapt to mice. This, of course, may be due to a virus strain difference rather than to variations in susceptibility of different litters to these agents.

TABLE I. Results of Inoculation of Suckling Mice with Stool Specimens Containing the Coxsackie Virus.

Stool specimen No.	Suckling mouse litter	Results	"C" virus infections
16	1	S, S, S, S, S, S, S, S	0/8†
	2	5,* 5, 5, 5, 6	5/5
186	1	S, S, S, S, S	0/5
	2	5, 5, 8, 8, S, S, S	4/7
	3	S, S, S, S, S, S	0/6
9	1	8, 8, 10, 10	4/7
	2	S, S, S, S, S, S, S, S	0/8

* Figure indicates day after inoculation symptoms observed. S animal showed no symptoms during observation period of 14 days.

† No. of mice which developed "C" virus infection/No. inoculated. (Animals missing, devoured or found dead within 48 hr after inoculation are excluded from analysis.)

Whether the observed differences are due to variability in litter susceptibility or to chance, it is obvious that some of these virus isolations might not have been successful if only one litter of suckling mice had been used. If the observed results are actually due to litter differences in susceptibility, it should be possible by selective breeding to develop a strain of mice of more uniform susceptibility as has been the case with other agents(8).

Summary. Data are presented which sug-

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gest differences in susceptibility of different litters of suckling mice used for the primary isolation of Coxsackie viruses. It is suggested that at least 2 litters including a total of 12 to 15 suckling mice be used for demonstrating Coxsackie viruses in clinical specimens.

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Augmentation by Pregnant Mares' Serum of Body Weight Response of Male Turkeys to Testosterone Propionate. (18778)

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In connection with experiments directed toward quite different ends, testosterone propionate has been observed to exert a greater effect on body growth of male turkeys when administered concurrently with pregnant mares' serum than in absence of this gonadotrophin. The capacity of androgens to stimulate somatic growth under certain conditions is well established(1-4), and testosterone propionate has been shown to act synergistically with pituitary growth hormone in this respect (5). Enhancement of the androgen-induced growth response by pregnant mares' serum, itself without effect on growth in these tests, seems however not previously to have been reported.

Materials and procedures. The experimental stocks consisted of 18 immature Broad Breasted Bronze and an equal number of mature Beltsville Small White male turkeys. The younger birds were 111 ± 7 days of age at the beginning of tests, close to 100 days from sexual maturity. The mature turkeys, 16 months old when placed under test, had entered the period of declining sexual activity some 3 months earlier, upon completion of their first breeding season. Seasonal quiescence in such males is associated with loss of body weight, and resurgent sexual activity with increasing weight(6). Insofar as latent capacity for increase in body weights is concerned, the immature and the mature males were thus much alike qualitatively.

The birds of all test groups were maintained under identical conditions; they were well housed, and had access to ample outside runs. Feed was available *ad libitum*. Treatment was carried out in 2 stages. During the first period of 22 days all the birds except uninjected controls received 7 injections of PMS, 200 rat units at each injection. At the termination of this period, testosterone propionate was administered to designated groups with and without continuing PMS injections; details are recorded in Table I. All birds were sacrificed at termination of secondary treatments, at which times their testes were removed and weighed. Body weights were taken at intervals from the beginning of tests until autopsy. The PMS* preparation, a solution containing 50 rat units per ml, was injected subcutaneously into the anterior cervical region. Testosterone propionate was dissolved in corn oil, 5 mg per ml, and was injected subcutaneously at alternate breast sites on successive days.

Results. The administration of PMS dur-

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* "Gonadin Serum," Cutter Laboratories, Berkeley, California. Crystalline testosterone propionate was generously furnished by the Research Department, Ciba Pharmaceutical Products, Summit, N. J.

TABLE I. Body and Testes Wt of Uninjected and PMS Control Turkeys, and of Birds Receiving TP or PMS + TP During Periods of Secondary Treatments.

No. Turkeys	Treatment*		Body wt				Wt of testes, g
	Primary 22 days	Secondary†	Initial, kg	Final, kg	Gain		
					kg	%	
			Immature males				
5	0	0	6.02	7.34	1.32	21.9	.85
5	PMS	PMS	6.64	7.86	1.22	18.1	2.46
4	”	TP, 2.5 mg	6.65	8.73	2.08	31.3	.77
4	”	PMS + TP, 2.5 mg	6.64	9.17	2.53	38.1	3.57
			Mature males				
4	0	0	8.82	8.84	.02	$<.01$	2.87
4	PMS	PMS	8.61	8.50	−.11	$<-.02$	5.40
5	”	TP, 10 mg	8.32	9.46	1.14	13.7	2.94
5	”	PMS + TP, 10 mg	8.37	10.02	1.65	19.7	2.05

* Treatment: Primary—PMS to avg 65 R.U. daily. Secondary—PMS, 200 R.U. every other day; TP daily as indicated.

† Periods of secondary treatment were 26 days for the immature males, 16 days for the mature males.

ing the period of pretreatment had little or no effect on body weights, a result in accord with earlier findings of Schooley, Riddle, and Bates(7).

Body weight responses under the several conditions of secondary treatment are summarized in Table I, together with testes weights at autopsy. Testes weights of the younger males at maturity and of the older males on resumption of sexual activity would have exceeded 25 or 30 g; the gonadal stimulation due to PMS treatment was accordingly moderate.

The potentiating action of PMS is best seen in Fig. 1 and 2. In the immature birds the effects of testosterone propionate and of the androgen plus PMS are of course superimposed on normal growth increases of considerable magnitude, and it is perhaps notable that rate of growth under PMS + TP (Fig. 1) almost exactly doubles this normal rate. Of more interest here, however, is the definitive augmentation by pregnant mares' serum, in the form used, of the androgen-induced increases in body weights, both in the immature and mature birds.

Discussion. Although pregnant mares' serum was without appreciable effect on body weights, we cannot assume that pretreatment with this preparation did not play some part in determining the subsequent responses to testosterone propionate, or to the androgen + PMS. It is to be noted also that augmentation of body weight responses to testosterone propionate was obtained with a preparation of pregnant mares' serum of known gonadotrophic potency, but containing much besides gonadotrophin; one may accordingly ask whether the observed augmentation is to be ascribed to gonadotrophin or to some other substance carried by the serum. Experiments designed to clarify these points are now in progress. There is little point in further com-

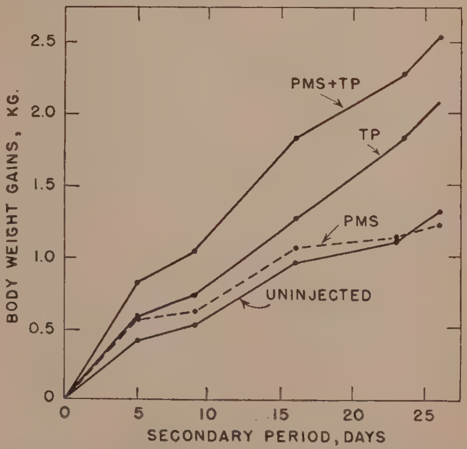

FIG. 1. Body wt gains of immature male turkeys following administration of testosterone propionate (TP), and of pregnant mares' serum with the androgen (PMS + TP); the lower curves show body wt changes of uninjected and PMS injected control groups.

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ment until the outcome of these tests is known, attractive though some possible implications of a gonadotrophically conditioned augmentation effect may appear to be.

Summary. Following preliminary treatment with pregnant mares' serum, body weight responses of immature and mature, but sexually inactive, male turkeys to testosterone propionate were considerably augmented when the androgen was administered concurrently with pregnant mares' serum. The serum alone was without appreciable effect on body weight. Whether the observed augmentation is an effect of gonadotrophin or of some other constituent of the serum is not known.

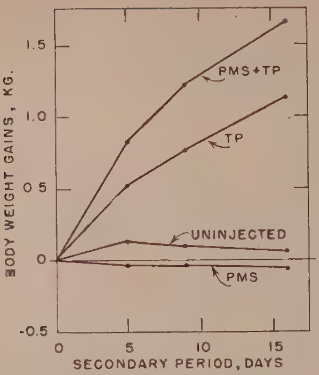

FIG. 2. Body wt gains of mature but sexually inactive male turkeys following administration of testosterone propionate (TP), and of pregnant mares' serum with the androgen (PMS + TP); also, body wt changes of uninjected and PMS injected control birds.

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Sustained Enhanced Growth of Carcinoma EO771 in C57 Black Mice.

(18779)

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Injection of lyophilized or long frozen Eo-771 tumor tissue 10-15 days prior to the transplantation of the same tumor renders heterologous(1,2) and homologous(3) hosts more susceptible to the growth of the homologous tumor transplants. This lyophilization(1,2) or XYZ factor(4) has been shown to be specific(3). Previous reports have not considered whether the tendency to enhanced growth induced by the XYZ factor might persist through successive transplant passages from such a treated animal. The present paper reports 17 experiments with 2-6 animal passages of the tumor from an XYZ injected animal, and compares the growth of the secondary passage tumors with a series of control and a series of first passage XYZ tumors.

Materials and methods. The animals employed were 529 C57 Black mice received from Carworth Farms† in batches of 30 and 60 over an 18-month period. Each batch was of same age and sex and were divided immediately into groups of 10, numbered and housed in standard mouse boxes for 1-4 months before use. The animal quarters were air conditioned and maintained at constant temperature, humidity and lighting and a standard food pellet was given. The control and experimental animals were always from the same batch. Four strains of C57 Black mammary carcinoma Eo771, designated A1, A2, A3 and A4, were obtained from 4 mice, 2549, 2550, 3749, and 3750, the first 2 received in one shipment in 1949 and the latter 2 in 1950 through the courtesy of Dr. George Snell of the Jackson Memorial Laboratory of Bar Harbor, Maine. Transplantation was effected by removing tumor tissue (15-27-day-old growth) aseptically from a subcutaneous growth which had not ulcerated. The tumor tissue was divided into 3 portions: (1) for histopathologic study, (2) for placing in the deep-freeze at 0°F in a sealed sterile container 10-90 days for XYZ material, and (3) for immediate transplantation. The latter was emulsified using a scissors, mortar and pestle and (1) sterile normal saline or (2) usually penicillin buffered with sodium citrate. About 0.1 cc of a 25% emulsion was injected subcutaneously into the left groin. (In some instances most of the tumor emulsion leaked out the needle hole in the skin.) There were usually 2 boxes (10 mice to a box) of control and 2 boxes of experimental mice in each experiment. The tuberculin syringes (one for each set) were filled at the same time. The order of inoculation was (1) control, (2) experimental, (3) experimental, (4) control box or (1) experimental, (2) control, (3) control, (4) experimental box. The XYZ material was frozen tumor tissue which had been kept in the deep freeze at 0°F in a sterile container for 10 days or longer, and was prepared and injected in an entirely similar manner. The injections of frozen tumor tissue were usually into the opposite groin 7-34 days before the tumor transplantation. No tumor growths or palpable inflammatory reactions appeared. The XYZ material was prepared from Eo771 tissue, and XYZ control material was similarly prepared and injected into 73 mice: (1) from Barrett mammary carcinoma(3) of C3H origin adapted to C57 blacks (10 mice), (2) from mammary carcinoma 241-5 (22 mice; carcinogen-induced tumor from Dr. Andervont's(3) laboratory), or (3) mammary carcinoma 755(3) (41 mice), the latter 2 being of C57 black origin (Table I).

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† Carworth Farms, New City, N. Y.

TABLE I. Mammary Carcinoma EO771 in C57 Black Mice: Controls, First and Multiple Passage XYZ Series.

		Control mice				
Exp. #	St.	Inoc. #	Pos. #	M. day	Σx	Σx^2
231	A1	10	10	20	27	102
245	A1	10	6	19		
246	A1	8	8	19	53	302
263	A1	10	6	19		
264	A1	10	10	19	75	459
268	A1	10	8	21	17	58
273	A1	8	8	15	26	110
288	A1	9	8	14	4	4
300	A1	10	7	18	21	102
318	A1	8	7	18	29	153
322	A1	10	9	21	25	90
324	A1	6	6	19	15	47
Subt.		109	93		292	
	Mean	109			2.68	
356	A3	10	8	20	13	37
357	A4	8	7	21	37	352
360	A3	10	10	20	19	64
368	A3	10	10	20	45	267
349	A3	9	8	21	15	48
376	A3	11	6	20	12	36
378	A3	9	9	20	40	282
380	A3	10	7	21	12	34
382	A3	9	6	21	20	109
383	A3	9	7	21	25	140
384	A3	11	10	21	26	96
Subt.		106	88		264	
	Mean	106			2.49	
225	A1^{B}	10	9	20	24	108
261	A1^{S}	14	13	19	65	452
266	A1^{S}	8	6	21	11	24
297	A1^{7}	9	5	18	4	4
Subt.		41	33		104	3480
	Mean	41			2.54	
Total		256	214		660	
	Control		Mean	256	2.58 ± .17	

				Experimental mice				
Exp. #	XYZ	Int. day	St.	Inoc. #	Pos. #	M. day	Σv	Σv^2
241	A2	25	A1	10	10	19		
243	A2	25	A1	9	9	19	135	1352
274	A1	18	A1	10	10	15	63	472
290	A1	19	A1	10	9	14	5	4
323	A1	13	A1	10	9	21	13	26
325	A1	15	A1	7	7	19	22	118
354	A1	8	A3	11	10	20	41	224
355	A1	14	A4	9	4	21	7	13
366	A1	8	A3	11	10	20	47	253
379	A3	8	A3	10	6	21	25	122
381	A3	8	A3	10	5	21	7	15
385	A3	8	A3	10	10	21	32	173
386	A3	8	A3	12	10	21	63	612
Total				129	109		460	3384
XYZ		First passages		Mean	129		3.57 ± .32	
337			A1X3	10	9	21	59	547
338			A1X3	7	7	19	11	32
334	755	7	A1X3	9	9	21	69	722
339	755	7	A1X3	10	10	19	44	288
Subt.				36	35		183	
			Mean	36			5.1	

TABLE I (continued)

335	A1	7	A1X3	7	7	21	58	1078
336	A1	7	A1X3	9	8	21	52	606
340	A1	9	A1X3	10	10	19	62	424
341	A1	9	A1X3	10	7	19	56	665
367	A1	8	A1X6 A1X4	10	8	20	16	54
Subt.				46	40		244	
			Mean	46			5.30	
328			A1X2 A1X4	7	6	18	21	77
345			A1X2 A1X4	9	9	18	55	389
352			A1X3 A1X5	7	7	21	79	1015
361			A1X4 A1X6	8	8	20	46	490
369			A1X4 A1X6	8	7	20	38	275
333			A1X3 A1X5	10	10	22	55	384
348	755	7	A1X3 A1X5	8	7	18	9	19
350	755	7	A1X3 A1X5	5	5	21	30	177
Subt.				62	59		333	
			Mean	62			5.37	
Total				144	134		760	
XYZ	Multiple passages				Mean	144	5.28 ± .37	

Exp.—experiments; St.—strain; Inoc.—inoculated; Pos.—grew tumors; M.—tumors measured; x—sum of tumor volumes; x^2—sum of squares of tumor volumes; Subt.—subtotal; XYZ—strains source for XYZ material; Int.—interval between XYZ injection and tumor transplantation; A1, A2, A3, A4—Bar Harbor strains of Eo771; $A1^B$, $A1^S$, $A1^7$—mice preinjected with Barrett ca. XYZ, 241-5 XYZ, and 755 XYZ respectively; A1X3, 4, 5, 6—tumor strain A1X 2, 3, 4, 5, or 6 mouse passages removed from an XYZ injected animal.

All experiments with Eo771 during 1949-50 are included in the study except certain stock transfers in which the only tumors measured were those used for transplantation or for XYZ material. There were 256 control mice, 129 mice in the first passage XYZ series, and 144 mice in the multiple passage XYZ series (Table I).

The 256 *control* mice were from 3 groups of experiments: (1) 109 mice in 12 experiments inoculated with the A1 strain of Eo771 mammary carcinoma over a 7-month period, (2) 41 mice in 4 experiments inoculated with the A1 strain of Eo771 several weeks after preliminary treatment with XYZ control material from the Barrett 241-5 or 755 mammary carcinomata, and (3) 106 mice in 11 experiments inoculated with the A3 and A4 strains of Eo771 beginning about one year after the start of the A1 strain experiments.

The 273 *experimental* mice were in 2 categories: (1) 129 mice in 13 experiments injected with Eo771 XYZ material 8-25 days before tumor transplantation (Table I); (2) 144 mice in 17 experiments in the multiple passage XYZ series (Table I). The Eo771 tumor strain used in these 144 mice was a variety of the A1 strain called A1X because of its having originated in an A1-Eo771 XYZ injected A1-Eo771 tumor animal 2-6 mouse passages earlier. This is the only instance in which secondary animal passages have been made from an XYZ injected mouse. (The rule has been to make transfers from control mice only.) The original mouse,[‡] No. 3253, from Exp. 325 (Table I) had at 20 days after transplantation (35 days after XYZ injection) a primary tumor in the groin which measured 3.45 cc (2 other animals in the group had larger tumors and 2 almost as large), and at 27 days when transfer was made the primary tumor measured 13.5 cc. (Volume of a tumor in our experiments is artificial, being the product of measurements in cm in 3 dimensions with calipers; measurements after tumor transplantation are routinely made at 19-20 days for Eo771 because deaths from tumor growth or ulcerations begin to occur in some animals at 21 days and thereafter.) Thus the tumor cells injected into each of the 144 mice in the 17 experiments traced back to tumor cells which grew in XYZ injected Mouse 3253. In addition the mice in Experiments 335, 336, 340, 341 and 367 also had an injection of A1-Eo771 XYZ material, and the mice of Exp. 334, 339, 348, and 350 had an injection of XYZ control material from 755 mammary carcinoma tissue (Table I). Among the 144 mice 36 represented the 3rd mouse passage from the original XYZ animal and were otherwise entirely like the controls; 46 of the 144 mice in 5 experiments (Exps. 355, 336, 340, 341, 367) were injected with the A1X tumor strain, and had, in addition, a preliminary injection of A1 XYZ material; the remaining 62 in 8 experiments injected with the A1X tumor strain had in addition a second injection of A1 XYZ two or three animal passages back.

[‡] From the tumor tissue in Mouse 3253 a stock transfer was made into mice 3202 and 3203 and 8 others (Exp. 320); from tumor growth in 3203 the mice in Exps. 338, 339, and 340 were injected; from the tumor growth in Mouse 3202, animals in Exps. 334, 335, 336, 337, and 341 were injected; from Mouse 3369 in Exp. 336, animals in Exp. 328 were transplanted; from Mouse 3286 in Exp. 328 were transplanted the animals in Exp. 345, 348 and from Mouse 3287 the animals in Exps. 353, 350, and in stock transfer Exp. 353 were transplanted; from Mouse 3531 of the stock transfer the mice in Exp. 333 were transplanted and from the tumor in Mouse 3339 of Exp. 333 the mice in Exps. 361, 369, and 367 were transplanted.

Results. The average volume of the primary tumor at 19-20 days among the entire 256 control mice was 2.58 ± 0.17 cc (variance 6.741; variance of the mean 0.0272). In 12 experiments 109 control mice inoculated with the A1 tumor strain had at 19-20 days an average primary tumor of 2.68 cc; in 11 experiments with the A3 and A4 strains, 106 control mice had an average primary tumor size at 20 days of 2.49 cc; and in 4 experiments in which 41 control mice, although inoculated with A1 strain, had preliminary injections of XYZ material from the Barrett, 241-5 or 755 mammary carcinoma strains, the primary tumor at 19-20 days averaged 2.54 cc. There was, therefore, no statistically significant difference between the growth of the controls for the A1 strain, treated or untreated with heterologous XYZ material, and the A3 and A4 strains. In fact, the average size of the tumors was almost identical in the 3 groups.

In the 13 experiments in which 129 mice were inoculated with the A1, A3, and A4 strains, but had received preliminary injections of Eo771 XYZ, the average volume of the primary tumor per animal inoculated was 3.57 cc at 19-20 days as compared with 2.58 cc among the 256 control mice. This difference of 0.99 ± 0.28 cc ($t = 3.5$, $n = 383$, $P = 0.01-$) was statistically significant and indicated that the preliminary treatment with the homologous XYZ material was effective in increasing the average size of the tumor growths by about 40%. There was no statistically significant difference in the incidence of tumors at 19-20 days between the controls (214 or 83.6%) and the first passage XYZ series (109 or 84.5%) (Table I). The mean size of the tumors among the 214 controls was 3.1 cc and among the 109 first passage XYZ series 4.2 cc. The difference 1.14 ± 0.23 cc is highly significant ($t = 5.0$, $n = 321$, $P. = 0.01-$) and even more striking than when calculated for animals inoculated.

The 144 mice in the 3 groups (17 experiments) transplanted with the A1X strain had uniform mean tumor volumes at 19-20 days. The tumor mean for the 36 animals after the 3rd mouse passage was 5.1 cc, for the 46 given another XYZ treatment, 5.3 cc, and for the 62 serially transferred after a second and third XYZ treatment of the tumor cell strain, 5.37 cc; the differences were statistically insignificant between the groups of the A1X strain variously treated. The mean value for the entire 144 animals inoculated was 5.28 cc or twice that for the 256 controls (statistically significant difference) and some 50% more than the mice receiving the preliminary injection of the XYZ material in the first animal passage (statistically significant difference).

Among the 144 multiple passage XYZ mice 134 had primary tumors at 19-20 days (94%) as compared with 214 among the 256 control mice (84%), a difference which was statistically significant ($\chi^2 = 7.2$, $n = 1$, $P. = .01-$). The mean volume of the primary tumors among the 134 positive multiple passage XYZ animals was 5.7 cc at 19-20 days as compared with 3.08 cc among the 214 positive controls. The difference, 2.59 ± 0.36 cc, was statistically significant ($t = 7.2$, $n = 346$, $P. = 0.01-$).

The incidence of primary tumors was significantly less ($\chi^2 = 5.1$, $n = 1$, $P. = 0.023$; probably significant) among the first passage XYZ series (189 or 85%) than among the multiple passage XYZ animals (134 or 94%). The mean size of the tumors in the 109 positive first passage animals was 4.2 cc and among the 134 positive multiple passage animals was 5.7 cc. The difference, 1.45 ± .47 cc, was statistically significant ($t = 3.1$, $n = 241$, $P. = 0.01-$).

Discussion. The average size of the primary tumor was constant and uniform at 19-20 days in the Carworth Farms C57 black control mice with 2 different Eo771 strains A1 and A3 studied almost one year apart. This indicates a high degree of uniformity in the animal stock, experimental technic, tumor behavior and tumor strains in the control mice. The enhanced growth in the XYZ series is, therefore, all the more significant, and the results amplify and confirm those already published on the effect of the XYZ material in the homologous strain(3). Snell and his group have reported(1,2,5) an enhancing effect of lyophilized (or XYZ) material from Eo771 and 15091a on heterologous strains, which is even more striking than that obtained in this series. In fact, Dr. Snell might explain that the enhanced effect of the homologous material with the Carworth Farms C57 black strain was due to the fact that this strain was genetically somewhat different from the Bar Harbor C57 black strain in which the tumor strains A1, A2, A3, and A4 had been carried previous to use in our laboratory.

The most remarkable result was the apparently permanent enhanced growth of the A1X strain in the 17 experiments herein reported. This might indicate that the second and later passages of a tumor cell strain from an XYZ treated animal might give rise to larger average growths than would occur in the first passage strain. This might seem almost conclusive were it not that the 17 experimental groups of the A1X strain traced back to the same XYZ treated transfer animal (3253). No other experiments with secondary mouse passages are available. Whether this was a mutation—and some form of mutation or adaptation it seemed to be—or a regular occurrence in XYZ injected animals, only further experiments can answer. Attempts to reverse the change are in progress.

Summary and conclusions. 1. Mammary carcinoma Eo771 was inoculated into 529 C-57 black mice in three series of experiments. Tumor growth in 109 control mice (12 experiments) injected with one Bar Harbor Eo771 mammary carcinoma strain averaged 2.7 cc at 19-20 days, and 41 control mice of the same strain (4 experiments) previously injected with heterologous XYZ material averaged 2.5 cc at 19-20 days. An additional 106 control mice in 11 experiments injected with a second Eo771 strain received from Bar Harbor about 1 year later averaged 2.5 cc at 20 days, indicating great uniformity in 2 different Bar Harbor strains of this tumor in Carworth Farms C57 black mice, there being no statistically significant differences. 2. In contrast, 129 C57 black mice in 13 experiments having a preliminary injection of Eo771 XYZ material 8-25 days before tumor transplantation had a 40% increased tumor volume at 19-20 days, or 3.6 cc. This difference of 0.99 ± 0.28 cc was statistically significant. 3. In an additional 17 experiments during the same period involving 144 mice of the same strain, mean tumor growth in 2-6 animal passages of tumor from an XYZ injected mouse averaged 5.3 cc or more than double the growth of Eo771 in control mice and some 40-50% greater than occurred in the first passage XYZ strains. Whether this greatly enhanced growth was a mutation or a variation induced by the XYZ treatment remains to be determined.

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Effect of Cortisone on Serum Non-Specific Hyaluronidase Inhibitor Following Surgical Trauma. (18780)

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Investigators have recognized for some time the fact that the serum non-specific hyaluronidase inhibitor is elevated in many pathological and pathophysiological states. This elevation of the inhibitor has been observed in experimental animals following trauma(1,2) and in patients with active rheumatic fever(3), acute lupus erythematosus (4), and certain neoplastic lesions(5). When ACTH and cortisone were added to the therapeutic armamentarium for the treatment of some of the so-called "collagen diseases", it became of interest to determine what alteration might occur in the serum hyaluronidase inhibitor in patients with elevated inhibitor values undergoing treatment with ACTH or cortisone. Recently Adams and co-workers (6) submitted data which demonstrated a fall in the serum nonspecific hyaluronidase inhibitor in patients with active rheumatic fever receiving ACTH therapy. The decrease in the titer of the serum inhibitor paralleled the clinical improvement observed in these patients.

It was the purpose of the experimental work reported in this paper to determine if the elevation of the serum non-specific hyaluronidase inhibitor noted in animals following wounding was modified by cortisone administration. That a possible interaction between cortisone and the inhibitor might exist in wounding is suggested by the work of Ragan *et al.*(7) who observed a suppression of granulation tissue in healing wounds when cortisone was administered in large doses although aseptic surgical wounds healing per primam are apparently little affected when amounts of cortisone commensurate with those used in humans are employed(8-10).

Method. Healthy mongrel dogs were used throughout the experiment. They received a standard kennel ration. Four animals received cortisone and 3 served as controls. The cortisone was administered subcutaneously beginning one day prior to wounding and daily until the twentieth day following the initial surgical incisions. The dosage of cortisone was 2 mg per kilo of body weight. The wounds were made under aseptic conditions on the animal's back under intravenous nembutal. They consisted of linear incisions carried down through all tissue layers to the periosteum of the ribs and were sutured with interrupted No. 40 cotton sutures. Wounding was repeated on the third, seventh, and tenth day following the initial trauma. Blood samples were drawn prior to the initial wounding for determination of the serum inhibitor and 3 times each week thereafter during the period of experimental observation. The blood was taken from the external jugular vein and placed in sterile tubes and allowed to clot. The blood was then centrifuged and the serum decanted into sterile tubes and stored at –20°C until the determination of the inhibitor titers was

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made. Freezing of the samples until a complete series can be run has the advantage of doing the determinations of the various samples with the same hyaluronic acid substrate and the same enzyme solution, thereby avoiding day to day changes in concentration which may occur with storage of these solutions. Determination of the serum inhibitor was made viscosimetrically using the method of Hadidian(11) with minor modifications. All determinations were made employing the Ostwald type viscosimeter with a flow time of 25 seconds for 4 ml of distilled water and carried out in a constant temperature water bath at 25°C ± .05°C. Care was exercised to suspend the viscosimeter in the water bath in the same position for all readings. 0.1 ml of serum was used for the determinations. The serum non-specific hyaluronidase inhibitor values were expressed as per cent inhibition using the formula suggested by Wattenberg and Glick(12).

Results. The serum inhibitor values in the cortisone treated animals and the control animals showed a similar response to the repeated trauma varying only slightly in degree. The serum inhibitor increased postoperatively to a peak value which occurred between the seventh and twelfth day following the initial trauma and fell to pre-operative values between the seventeenth and twentieth postoperative day. (Fig. 1). An exception arose in the case of a control animal in which the titer remained elevated above the normal range on the twentieth day of the experiment.

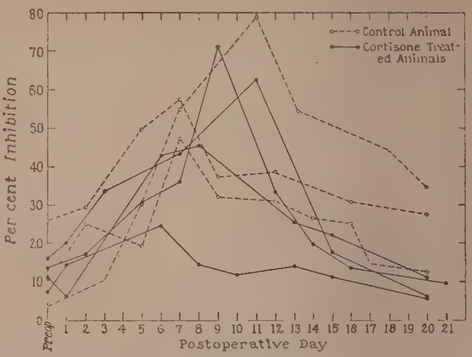

FIG. 1. Graph demonstrating per cent inhibition of hyaluronidase activity by serum of experimental and control animals during postoperative state.

One of the animals receiving cortisone showed a similar rise in the inhibitor following surgery but the level, although significantly elevated, did not attain the height observed in other experimental or control animals. Failure of cortisone to alter the inhibitor has been similarly observed by Glick(13).

Summary. Following repeated wounding of healthy mongrel dogs there is a rise in the titer of the serum non-specific hyaluronidase inhibitor which tends to return to normal preoperative values as healing occurs. This response of the inhibitor is unaltered by the administration of cortisone under the conditions of this experiment.

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Sensitivity Studies *in vitro* with *Histoplasma capsulatum* to Various Potential Therapeutic Agents. (18781)

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During recent years histoplasmosis as a significant clinical entity has been brought to the fore. The infection usually appears in the benign form, however, the fulminating type of the disease is more important as a clinical entity. Many drugs and antibiotics have been used in the attempt to treat this infection but without avail.

Material and methods. On February 21, 1949 an infant with histoplasmosis was admitted to the Children's Hospital. The patient was a 6½-months-old white female whose chief complaints were fever, cough and enlarged abdomen. The child had been hospitalized previously in another city and was referred to us with the diagnosis of acute lymphatic leukemia. A bone marrow biopsy was performed and the presence of intracellular yeastlike cells in the macrophages led to the diagnosis of histoplasmosis. Cultures of blood and bone marrow yielded *Histoplasma capsulatum*. Penicillin, aureomycin and sulfadiazine were administered, and general supportive therapy including repeated transfusions was instituted. The patient expired approximately 2 months after onset of the illness, which was 2 weeks after admission to the hospital. The organism isolated from the bone marrow of this patient was maintained in the yeast phase by frequent transfer on blood agar slants. Since the organism occurs in the infection in the yeast form, it is necessary that inhibition studies be conducted with this phase. By transferring at weekly intervals the culture grew rapidly, producing heavy growth within four days. *In vitro* studies were conducted using the gamma and delta isomers of benzine hexachloride, quinine bisulfate, atabrine, emetine, aminopterin, 2,4-dichlorophenoxyacetate, para-aminosalicylic acid, urethane, and ethyl vanillate. The benzine hexachloride isomers, aminopterin, urethane and para-aminosalicylic acid were incorporated in graded concentrations in blood agar using 5% human blood in the agar base described by Casman(1). The remainder of the materials were tested in a liquid medium described by Salvin(2). Although this medium contains no blood or serum, the organism is maintained in the yeast phase. The cultures were inoculated with a light suspension of *Histoplasma capsulatum*. The number of viable cells inoculated was not determined since the size inoculum did not significantly alter the sensitivity results. All cultures were sealed with parofilm to maintain the moisture and were incubated at 37°C. The first readings were made after 5 days incubation and final observations were made when the tests were discarded after 3 weeks.

TABLE I. Sensitivity Studies *in vitro* with *Histoplasma capsulatum* to Various Potential Therapeutic Agents.

Test material	Inhibited by, mg %	Tolerated, mg %
Gamma benzine hexachloride	10	5
Delta ” ”	10	5
Quinine bisulfate		3.2
Atabrine	3.3	.33
Emetine	3.2	.32
2, 4 dichlorophenoxyacetate		100
Para-aminosalicylic acid		200
Aminopterin		.1
Urethane		100
Ethyl vanillate*	50	40

* Obtained from The Institute of Paper Chemistry, Appleton, Wisc.

Results. The results of the *in vitro* studies are summarized in Table I.

The isomers of benzine hexachloride inhibited the test organism in a concentration of 10 mg %. These materials, known primarily as insecticides(3) have been found inhibitory for certain micro-organisms, including various yeasts and the tubercle bacillus, however, their

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use as a potential therapeutic agent is quite limited because of the toxic properties. When mixed with blood or plasma these isomers are bound to the protein and have been found to be without value in the treatment of experimental tuberculosis in mice and guinea pigs(4).

Quinine bisulfate, used in the treatment of malaria, failed to inhibit growth in a concentration of 3.2 mg %, the maximum concentration tested.

Atabrine, another antimalarial drug, prevented the growth of the organism in a concentration of 3.3 mg %. This drug is deposited in high concentrations in the reticulo-endothelial system and as reported elsewhere, has been active *in vitro* against *Histoplasma capsulatum*. Saslaw, Campbell, and Kuhn(5) reported that when administered early in the infection in mice, the mortality was reduced, however, atabrine was not effective once the disease was well established.

Emetine exhibited the same inhibitory concentrations as atabrine. This drug as used in the treatment of amebiasis is quite toxic to the myocardium, hence the potential application therapeutically is limited.

Because of its ring structure 2,4-dichlorophenoxyacetate (2,4-D), a selective herbicide(6), was included in the test materials. There was no inhibition at a concentration of 100 mg %.

Growth of *Histoplasma capsulatum* was not inhibited by para-aminosalicylic acid in a concentration of 200 mg %. Although this compound has bactericidal activity against *Mycobacterium tuberculosis*, its primary value has been the retardation of the development of streptomycin resistance in the tubercle bacillus(7).

Aminopterin, a biologic folic acid antagonist, failed to inhibit growth in the maximum concentration tested, 0.10 mg %. This concentration is higher than the level obtainable in the body.

Urethane, which is known for its anesthetic and growth suppressive properties, has been studied as a possible therapeutic agent for leukemia. This drug failed to suppress growth in a concentration of 100 mg %.

Ethyl vanillate, inhibitory in a concentration of 50 mg %, appeared to be the most promising material tested for the treatment of histoplasmosis. This substance is a by-product in the manufacture of sulfite pulp. Although it is relatively toxic, therapeutic levels can be obtained. Christie and co-workers(8) in a recent report stated that they used this drug in the treatment of 12 patients with the progressive type of histoplasmosis. Five of their patients recovered. Since this form of the disease is usually fatal and some of their patients had well advanced infections, the authors considered the recovery rate to be of significance. Ethyl vanillate was administered as a 40% solution in olive oil with a maximum dosage of 1.5 g per kg body weight per day. A blood level of 20 to 30 mg per 100 ml was usually obtained on this dosage. On the basis of our findings, their organisms were more sensitive than the strain which we studied. At the same time, ethyl vanillate was the only agent studied which showed promise as a possible therapeutic agent.

Summary. *Histoplasma capsulatum* isolated from an infant who succumbed to the infection was tested *in vitro* in the yeast phase to various substances known to be harmful to certain protoplasmic structures. The test materials were gamma benzine hexachloride, delta benzine hexachloride, quinine bisulfate, atabrine, emetine, 2,4-dichlorophenoxyacetate, aminopterin, para-aminosalicylic acid, urethane and ethyl vanillate. Growth was inhibited by ethyl vanillate in concentrations therapeutically attainable in man.

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Effect of DL-Ethionine on Poliomyelitis Virus Growth in Tissue Culture.* (18782)

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The role of certain amino acids in the growth and multiplication of viruses *in vivo* is becoming more and more evident. Sprunt (1) found that mice fed a low protein diet showed an increased resistance to infection with swine influenza virus with a reversal of this effect when methionine was incorporated in the diet suggesting that methionine is essential for viral multiplication. Jones, *et al.*(2) noted that low protein or low tryptophane diets delayed symptoms of Lansing type poliomyelitis infection in mice during the first two weeks and Rasmussen *et al.*(3) also report a lengthened incubation period with tryptophane deficiency in mice and that the deficiency is aggravated by 6-methyl tryptophane, an analogue of this amino acid. Rafelson, Pearson and Winzler(4) have shown that large amounts of certain amino acids may interfere with the phosphorus metabolism of mouse brain tissue supporting the growth of Theiler's virus in tissue culture. The inhibitory effects of one of these, lysine, could be partially overcome by the addition of methionine. The importance of methionine in the biosynthesis of influenza virus has been demonstrated by Ackermann(5) who studied the effects in tissue culture. By incorporating structural analogues of methionine into the tissue culture medium he found that virus propagation was inhibited. Furthermore, this inhibitory effect could be reversed or blocked by the addition of methionine to the fluids. He concluded that the inhibitors do not act by destroying the virus, the host tissue, or by preventing infection of the cells, but by interfering with the synthesis of the virus.

The present study was undertaken to determine the effect of dl-ethionine upon another virus, the Lansing strain of poliomyelitis, cultivated in tissue culture.

Experimental. A line of mouse adapted Lansing strain of poliomyelitis virus which has been maintained in this laboratory was used to initiate infection of the tissue. The mouse infectivity of the original pooled virus suspension was 10^{-4}. Virus was grown *in vitro* using the tissue culture technic described by Enders, Weller and Robbins(6). Brain tissue from human embryos of 3 to 4 months was minced and distributed under perforated cellophane in 25 ml flasks. Each flask then received 3 ml of Hank's solution plus Simms ultrafiltrate, to which had been added penicillin (90 u/ml) and streptomycin (0.1 mg/ml).

The flasks were tightly stoppered and incubated at 37°C. When the pH decreased to approximately 6.7 as indicated by a small amount of phenol red in the fluids, they were removed and fresh fluid at pH 7.2 was added, usually at intervals of 2-4 days. 0.1 ml of virus was added at the first fluid change and the titer of virus in subsequent fluid changes was determined by mouse infectivity tests in groups of 8 mice per dilution. The virus for subsequent experiments was always from a previous tissue culture, and the titer of the fluid at the start of each experiment was

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* Aided by a grant from the National Foundation for Infantile Paralysis.

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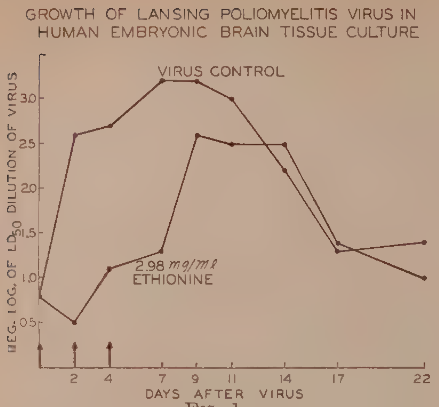

FIG. 1.

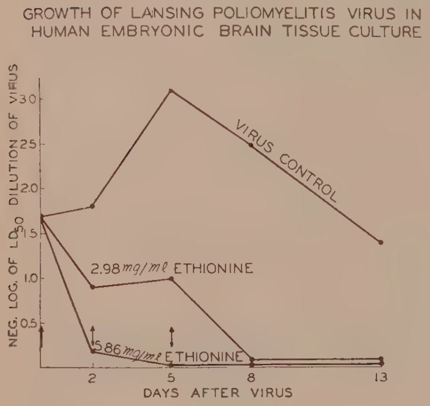

FIG. 2.

recorded as 1/30 (0.1 in 3 ml) of the amount of virus added.

Results. The peak of the virus titer, 10^{-3} to $10^{-3.3}$ was always obtained between the 4th and 7th days after addition of virus to the culture, but significant quantities were present between the 2nd and 11th days. It was observed that the best growth curves were obtained with tissue of embryos most recently viable.

Fig. 1 shows a typical growth curve and also the effect of ethionine on virus propagation. When 2.98 mg/ml were added to some of the flasks concurrently with the virus and again 2 and 4 days later at the 1st and 2nd fluid changes, a marked suppression of viral growth was detected. The tissue, however, was still capable of supporting multiplication of the virus as evidenced by a distinct increase in virus titer when ethionine was withheld from subsequent fluid changes.

Fig. 2 shows another experiment in which two different quantities of ethionine were employed. When the original concentration was doubled there was almost complete inhibition of virus.

In order to determine the effect of ethionine on the virus itself 3 ml of Hank's solution plus 0.1 ml of tissue culture virus having an infective titer of $10^{-3.2}$ were incubated at 37°C with and without ethionine (6 mg/ml). These conditions exactly duplicated the tissue culture technic except for the absence of living tissue. In addition, 2 ml of the same preparation of virus was allowed to stand at 37°C in the presence and absence of ethionine. The various mixtures were titrated in mice after 18 and 44 hours. The results presented in Table I show that ethionine has no destructive action on the virus itself under these conditions.

If the action of ethionine is specifically directed against its analogue, methionine, the addition of the latter to cultures containing ethionine should result in reversal of the inhibition. To test this hypothesis the experiment portrayed in Fig. 3 was conducted. In agreement with the results of previous tests, the growth curve of the virus in the control flasks was much higher than in the flask containing ethionine. The flasks containing ethionine together with methionine showed an intermediate growth curve, by no means as high as in the controls, but considerably higher than when ethionine alone was used. This demonstrates that under these conditions methionine limits the inhibitory effect of ethionine; further experiments using other proportions of the two compounds are being conducted in order to determine the optimal quantitative relationship. In an effort to

TABLE I. Effect of DL-ethionine on Mouse Infectivity Titer of Lansing Poliomyelitis Virus Incubated at 37°C.

	18 hr	44 hr
Hank's sol'n 3 ml + virus .1 ml	$10^{-1.1}$	$10^{-.1}$
Hank's + virus + ethionine 6 mg/ml	$10^{-1.1}$	$10^{-.6}$
Virus 2 ml	$10^{-2.4}$	$10^{-2.1}$
Virus 2 ml + ethionine 6 mg/ml	$10^{-2.2}$	10^{-2}

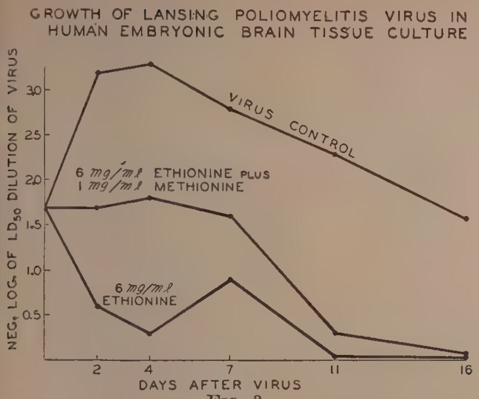

Fig. 3.

demonstrate true reversal of ethionine effect, methionine was added after several fluid changes to flasks already showing ethionine inhibition. No observable increase in virus growth took place but the significance of this experiment is rendered doubtful by the fact that cultures were in a state of deterioration at the time.

Attempts were made to substitute betaine and homocysteine for the methionine in the above experiment but the viral growth curve closely paralleled that with ethionine alone indicating that embryonic brain tissue is not able to synthesize methionine under these conditions from metabolites which in other biological systems are its immediate precursors (7).

Summary. 1. DL-ethionine inhibits the growth of Lansing strain of poliomyelitis virus in tissue cultures of human embryonic brain. 2. When the inhibitor is withheld from subsequent fluid changes virus growth is resumed indicating that the tissue itself is not irreversibly affected. 3. The effect of ethionine was shown not to be on the virus itself under conditions identical in all respects except for the absence of living tissue. 4. The inhibitory effect of ethionine was reduced by the addition of methionine to the cultures.

Conclusions. The Lansing strain of poliomyelitis virus requires L-methionine for its growth and multiplication in cultures of human embryonic brain tissue. Its growth can be materially inhibited with DL-ethionine, a structural analogue of the essential amino acid. The inhibitor acts by interfering with the biosynthesis of the poliomyelitis virus just as it suppresses the growth of influenza virus. This technic affords a procedure for studying the growth requirements of poliomyelitis virus and for testing materials which may influence its growth.

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A Non-Particulate, Dinitrocresol-Resistant, Glycolytic, Phosphorylating Mechanism Present in Malignant and Certain Normal Tissues. (18783)

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The present experiments deal with the respective abilities of cell-free fractions from normal and tumor tissues to derive energy for phosphorylation from oxidation of succinate or from glycolytic breakdown of hexose diphosphate. Cell-free, particulate systems from a number of normal tissues can apparently utilize up to 50 or 60% of the energy derived from oxidation of α-ketoglutarate, glutamate, or succinate for synthesis of phosphate compounds(1,2), which are in turn available for other cellular syntheses. These oxidative, phosphorylating, particulate systems have been shown by Loomis and Lipmann(3) and by Cross *et al.*(4) to be highly sensitive to the

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action of dinitrophenol and other nitro- and halophenols.

Warburg(5) showed that slices of tumor tissue exhibited a higher anaerobic and aerobic lactic acid production than most normal tissues. The Coris(6) demonstrated that high lactate production occurred also in tumors *in vivo*. From these observations arose the view that lactate production, by essentially anaerobic processes, could serve as a source of energy for function, *i.e.* growth of the tumor. Provisional support for this idea was provided by the repeated finding that certain oxidative enzymes, particularly succinic oxidase, were deficient in tumors(7), but comparatively little quantitative evidence as to the relative importance of the aerobic and anaerobic processes for energy utilization in tumors has been available. LePage and his associates (8-10) have studied anaerobic phosphorylation and glycolysis in malignant and other tissues and have shown that tumor and brain homogenates can give a positive phosphorus uptake under strictly anaerobic conditions. They did not apparently determine whether this anaerobic phosphorylation was affected by dinitrophenol.

Materials and methods. Liver and brain were obtained from normal albino rats weighing approximately 150-200 g. Walker carcinoma 256 was maintained by transplantation in rats of the same strain, weight, and age. The rat for each experiment was decapitated and the liver, brain, or tumor removed and placed immediately in 500 ml ice-cold KCl medium (0.12 M KCl buffered with 0.024 M glycylglycine to pH 7.2). All subsequent procedures were conducted at 4°C until introduction of the Warburg vessels into the bath for incubation at 25°C. After 5 minutes in the cold, buffered KCl medium, the tissues were removed, blotted to remove excess fluid, weighed, and homogenized with 2-3 volumes of the KCl medium. Liver or brain was hand ground in an ice-cold mortar with pestle for 2 minutes, half the KCl medium was added, the tissue reground for one minute, the rest of the KCl medium added, and the mixture reground for another minute. The resulting homogenate was brought up by addition of the KCl medium to a volume such that the materials from 250 mg original tissue were contained in 1 ml of the homogenate. The tumor, after removal of hemorrhagic and necrotic areas, was homogenized and handled in the same manner except that the Potter-Elvehjem homogenizer was used instead of the mortar. Each homogenate was filtered through cheesecloth to remove strands of tissue and inadequately dispersed materials. Separation into particulate and supernatant fractions was accomplished by centrifuging in certain experiments at 2,000 g for 15 minutes and in other experiments at 20,000 g for one-half hour, in all cases at a temperature of 4°C. In the experiments in which centrifuging was carried out at 20,000 g, the sedimented particles were made up to the original homogenate volume with the KCl medium and recentrifuged, the wash discarded, and the particulate material resuspended in the same volume of KCl medium. In the experiments in which centrifuging was carried out for 15 minutes at 2,000 g, a second centrifuging was carried out, the second wash discarded, and the particulate material resuspended in the same volume of KCl medium. In both cases the original supernates were used without further treatment.

The chilled Warburg flasks received additions in the following order: *Main compartment*: 1.3 ml of a solution to give the following final concentrations in a total volume of 3 ml; glycylglycine (pH 7.2), 0.025 M; substrate (succinate or pyruvate), 0.01 M; $MgCl_2$, 0.0066 M; KF, 0.0133 M; fructose, 0.0167 M; $KHCO_3$, 0.0066 M; nicotinamide, 0.04 M; diphosphopyridine nucleotide, 0.0002 M; adenosine triphosphate, 0.00033 M; hexose diphosphate (where present), 0.002 M; 0.1 ml yeast hexokinase; 1 ml homogenate,

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TABLE I. Phosphorus Uptake and Lactate Production of Walker 256 Rat Tumor under Aerobic and Anaerobic Conditions. Homogenate of Walker 256 rat tumors and particulate and supernate obtained from tumor homogenate by centrifuging at 20000 g for 30 min. Incubated 40 min. at 25°C. Substrate: hexose diphosphate and pyruvate. Each experiment is based on material derived from 250 mg original tissue and is the avg of 3 determinations. All results are expressed in micromoles.

	P uptake		Lactate formed		
Preparation	In air	In 95% N_2–5% CO_2	In air	In 95% N_2–5% CO_2	O_2 uptake in air
Homogenate	11.1	6.6	9.9	7.7	2.5
Particles	3.3	1.9	4.8	3	1.1
Supernate	6.5	6.2	7.3	6.1	2

TABLE II. Avg Phosphorus Uptake, Lactate Formation, and Oxygen Uptake of Homogenates of Walker 256 Rat Tumor, Rat Brain, and Rat Liver and Particulates and Supernates of the Same Obtained from the Homogenates by Centrifuging at 20000 g for 30 Min. Incubated 40 min. at 25°C. Substrate: hexose diphosphate and pyruvate. Each experiment is based on material derived from 250 mg original tissue and is the avg of 2 or 3 determinations. All results are expressed in micromoles.

Source of material	No. of exp.	Measurement made	Homogenate	Particles	Supernate
Walker 256 rat tumor	6	P uptake	14.6	1.6	8.9
		Lactate formed	9.5	2.7	7.3
		O_2 uptake	2.5	1.1	1.6
Normal rat brain	7	P uptake	17.8	7.8	12.3
		Lactate formed	5.3	2.1	6.8
		O_2 uptake	7.4	4.6	2.2
Normal rat liver	4	P uptake	18.1	11.8	6.9
		Lactate formed	2.8	.5	4.9
		O_2 uptake	10.4	8.1	4.1

particulate system, or supernate fraction (each derived from 250 mg original tissue); 0.3 ml water or 4,6-dinitro-o-cresol (DNC) solution to give the final concentrations shown in the tables. The DNC solution was added after the tissue fraction in order to utilize the well-known effect of substrate saturation preserving optimal activity of the enzyme as long as possible. *Side arm*: 0.3 ml 0.05 M Sorensen phosphate, pH 7.2; consequently with the additional phosphate introduced with the experimental materials the total amount of phosphate phosphorus initially present in each flask was approximately 500-600 μg. *Center cup*: 0.3 ml 5 N NaOH for aerobic experiments; unused for anaerobic experiments. The flasks, prepared as described, were attached to Warburg manometers, equilibrated in the bath for 3 minutes at 25°C, the taps were closed, phosphate tipped from the side arm, and the zero time reading for oxygen consumption taken. The flasks were shaken for 40 minutes, then removed to an ice bath. The flask contents were deproteinized by addition of an equal volume of ice-cold 20% trichloroacetic acid. Determinations of phosphate by the Fiske-SubbaRow and of lactate by the LePage method were made on the protein-free filtrates from the incubated and also from correspond-

TABLE III. Phosphorus Uptake, Lactate Formation, and Oxygen Uptake with Succinate as Substrate in Homogenates from Walker 256 Tumor, Rat Brain, and Rat Liver and Particles and Supernates Obtained by Centrifuging the Homogenates for 30 Min. at 20000 g. Gas phase: air. Each experiment represents material derived from 250 mg original tissue. Incubation time 40 min. at 25°C. All results are expressed in micromoles.

Source of material	Measurement made	Homogenate	Particles	Supernate
Walker 256 rat tumor	P uptake	9.2	3.2	.2
	Lactate formed	1.3	0	–.5
	O_2 uptake	3.9	2.5	1.8
Normal rat brain	P uptake	16.1	8	1.3
	Lactate formed	1.4	.8	0
	O_2 uptake	8.8	6.6	.7
Normal rat liver	P uptake	17.8	15.1	2.9
	Lactate formed	.8	.4	0
	O_2 uptake	14.3	9.7	3.7

TABLE IV. Effect of Dinitrocresol on (1) The Oxidative, Particulate, Phosphorylating Systems (2) The Water-Soluble, Glycolytic, Phosphorylating Systems Obtained by Centrifuging the Homogenates of Walker 256 Rat Tumor, Rat Brain, and Rat Liver at 20000 g for 30 Min. Each experiment represents material derived from 250 mg original tissue. Substrates: succinate or pyruvate plus hexose diphosphate. Gas phase: air. Incubation time 40 min. at 25°C. All results expressed in micromoles.

		Substrate: Succinate			Substrate: Pyruvate + HDP		
	Conc. of DNC	P uptake	Lactate formed	O_2 uptake	P uptake	Lactate formed	O_2 uptake
			Walker 256 rat tumor				
Particles	0	0	0	1.8	2.9	3.8	1.3
	0	-.1	-.2	2.2	3	4.2	1.4
	10^{-5} M	-.9	.1	1.8	2.5	3.9	.7
	10^{-4} M	-.8	0	1.7	1.9	3.7	.9
	10^{-3} M	-1.1	.1	1.5			
Supernate	0	.2	.2	.9	8.6	7.9	1.5
	0	-.2	-.3	.7	8.2	7.6	1.2
	10^{-5} M	.4	.4	1.3	8.5	7.7	1.3
	10^{-4} M	.2	-.4	1	8.9	8.1	1.1
	10^{-3} M				10.5	8.5	1.1
			Normal rat brain				
Particles	0	12.9	.6	7.6	7.4	1.4	4.6
	0	13.6	2.2	7.2			
	10^{-5} M	0	.7	4.6	1.2	1.6	5.5
	10^{-4} M	0	1	4	1	1.7	4
	10^{-3} M	0	1.3	2.2	.6	1.5	3
Supernate	0	1.4	-.5	2.1	11.6	7.6	2.1
	0				10.2	7.1	2.1
	10^{-5} M	.7	-.3	1.8	13.1	8.3	1.1
	10^{-4} M	.5	-.4	2	12.3	8	2.2
	10^{-3} M	1.1	-.7	1.6	13.5	8.4	1.9
			Normal rat liver				
Particles	0	13.1	.6	9.6	9.2	1.1	8.8
	0	13.2	.5	9.8			
	10^{-5} M	-.6	.8	13.5	-.4	1.5	10.4
	10^{-4} M	-1.2	.8	12.1	-.5	1.4	5.7
	10^{-3} M	-.8	.7	7.4	-.7	1.3	3.9
Supernate	0	2.3	.2	1.9	7.8	7.8	2.4
	0				7.6	7.9	2.3
	10^{-5} M	2	-.1	1.9	8	8.5	2
	10^{-4} M	2.8	0	1.9	7.6	6.6	3.5
	10^{-3} M	2.4	.3	1.4	7.4	5.4	1.6

ing zero time control samples. All results in the tables represent gain or loss during the incubation period.

In preliminary measurements it was found that the aerobic phosphate uptake by liver particles prepared and tested by this procedure with succinate as substrate was very rapid; the average values for P uptake in micromoles, for oxygen uptake in microatoms, and for the P/O ratio were, respectively: 5 minute incubation, 10.2, 4.58, and 2.22; 10 minutes, 14.4, 9.04, 1.59; 20 minutes, 15.2, 14.74, 1.02; 40 minutes, 15.0, 25.22, 0.59. Since a 40-minute incubation period was used for all tabulated measurements, the P/O ratios are obviously lower than the optimal values obtainable over shorter periods. No measurements of the time course of the P uptake associated with lactate formation have been made. It should also be noted that phosphorus uptake, oxygen consumption, and lactic acid production are minimal values, since a certain amount of material has of necessity been lost in the various steps involved in the preparation of the fractions and no allowance has been made for such loss.

Results. The observations will be considered under two main headings.

1. *Phosphorylation associated with particle and supernatant fractions.* In the first experiment to be reported phosphorus uptake and lactic acid production of tumor homogenate, particles, and supernate were determined under aerobic and anaerobic conditions (Table

TABLE V. Effect of Dinitrocresol on Phosphorus Uptake, Lactate Formation, and Oxygen Uptake of Homogenates of Rat Liver, Rat Brain, and Walker 256 Rat Tumor and Supernates of the Same Obtained from Homogenates by Centrifuging at 2000 g* for 15 Min. Incubation 40 min. at 25°C. Substrate: hexose diphosphate and pyruvate. Each experiment is based on material derived from 250 mg original tissue. All results expressed in micromoles.

		Normal rat liver			Normal rat brain			Walker tumor 256		
Preparation	Conc. of DNC	P uptake	Lactate formed	O_2 uptake	P uptake	Lactate formed	O_2 uptake	P uptake	Lactate formed	O_2 uptake
Homogenate	0	19.2	4.5	9.8	18.4	7.8	7.6	10.9	9.6	3
	2×10^{-6} M	18.2	4.1	10.2	14.8	8	7.9	9.1	9	2.5
	8×10^{-6} M	9	4.7	10.3	11	8	8.6	8.8	10	2.1
	3.2×10^{-5} M	5.8	4.9	15.1	8.8	7.7	8.6	8.6	9.3	2.3
	5.12×10^{-4} M	4.6	4.5	13.3	8.3	7.9	6.2	9.7	11.1	2.3
Supernate	0	5.5	7.9	8.2	12.8	8.7	3.5	7.7	9.3	1.1
	2×10^{-6} M	6	8.5	7.3	12.9	8.1	3.2	8.1	9.3	.9
	8×10^{-6} M	5.8	8.6	7.9	15.4	9.7	3.3	8.2	9.2	1
	3.2×10^{-5} M	5.8	8.8	8.5	14.6	11.6	3.3	8.7	10.6	1.2
	5.12×10^{-4} M	6.1	7.7	9	15	11.3	3.4	9.1	9.8	1

* Centrifuging at 2000 g instead of 20000 g was used in this and the following experiment, as it had been found that when equal amounts of a given tumor homogenate were centrifuged (1) at 2000 g and (2) at 20000 g, the phosphorus uptakes of particles, after washing, were 1.2 and 1.3 micromoles and of supernates 9.9 and 10.5 micromoles, respectively, and that both supernates were unaffected by dinitrocresol. The separation of particles may have been facilitated by a slight jelly formation.

TABLE VI. Phosphorus Uptake and Lactate Production by Homogenate of Walker 256 Rat Tumor and Supernate Derived Therefrom by Centrifuging at 2000 g for 15 Min. Measurements were made under aerobic and anaerobic conditions with and without addition of dinitrocresol. Each experiment represents material derived from 250 mg original tissue. Incubation time 40 min. at 25°C. All results expressed in micromoles.

		P uptake		Lactate formed		
Preparation	Conc. of DNC	In air	In 95% N_2–5% CO_2	In air	In 95% N_2–5% CO_2	O_2 uptake in air
Homogenate	0	10.9	7.3	9.6	8.9	3
	3.2×10^{-5} M	8.6	7.2	9.3	8.4	2.3
	5.12×10^{-4} M	9.7	9	11.1	10.8	2.3
Supernate	0	7.7	7.2	9.3	8.2	1.1
	3.2×10^{-5} M	8.7	7.1	10.6	8.6	1.2
	5.12×10^{-4} M	9.1	8.1	9.8	8.3	1

I). The observation of Schneider and LePage that phosphorylation occurred under anaerobic conditions in the homogenate, particles, and supernate of tumor material was confirmed. In the aerobic series the sum of the phosphorus uptakes by particles and by supernate was 88% of the phosphorus uptake by the whole homogenate, in the anaerobic series 117%. Under anaerobic conditions the homogenate can account for 60%, and the combined fractions for 73%, of the total phosphorus uptake by the homogenate under *aerobic* conditions. Further, about 60% of the phosphorus uptake by the homogenate under aerobic conditions is accounted for by the anaerobic, water-soluble, glycolytic activities of the supernate fractions, which are, as might be expected, the same, within the range of experimental error, under both aerobic and anaerobic conditions.

In the second experiment to be reported the average capacities of homogenates and of individual fractions of homogenates of tumor, brain, and liver to effect phosphorylation, to form lactate, and to consume oxygen under aerobic conditions were determined (Table II). The phosphorus uptake of the homogenates from each of the three tissues is not greatly different: 14.6 micromoles for tumor, 17.8 for brain, and 18.1 for liver. However, that of the particles differs greatly: 1.6 micromoles for tumor, 7.8 for brain, and 11.8 for liver. For homogenate, particles, and supernate, the oxygen uptake is lowest with the tumor material, considerably higher with brain, and highest of all with liver. The lactate formation, on the contrary, is highest with the tumor, intermediate with brain, and lowest with liver.

It is clear that a substantial fraction of the phosphorylation in the tumor is associated with glycolytic processes and this is confirmed by the phosphate uptake found under anaerobic conditions previously reported. These results offer quantitative experimental support for the assumption widely held for over twenty years that the energy made available by lactate production in tumors can be used for synthetic processes, in this case production of phosphate intermediates which can lead to synthesis of organic phosphate esters.

In liver and brain the sum of the phosphorus uptakes of the particles and supernates was equal to or in excess of that of the whole homogenate. In the case of the Walker tumor the phosphorus uptake of the particles and supernates in relation to that of the homogenates varied greatly, possibly due in part to the varying state of development of the tumor tissues employed.

In this series of experiments the average phosphorus uptake of the tumor supernate was approximately 61% and that of the particles plus supernate 72% of that of the homogenate. This discrepancy is due in part to loss of the glycolytic phosphorylating mechanism in the wash liquors, which has been found to be as much as 30% of that present in the supernate. When a tumor homogenate was centrifuged, the phosphorus uptake and lactate production of the recombined, unwashed particles and supernate were over 90% of those of the original homogenate, from which it may be concluded that no serious injury of the phosphorylating mechanism results from centrifugation. Some loss of phosphate ester, due possibly to phosphatase action, undoubtedly occurs in connection with the tumor particles, which in certain experiments results in a negative phosphorus uptake, which never occurs with liver or brain particles. Considerable difficulty has been experienced in securing an adequate amount of tumor material to study the phosphorylating mechanism of tumor particles as compared with those of liver and brain particles. It is proposed to make this the subject of a future investigation. With brain and liver, the combined lactate production of the individual particle and supernate fractions was considerably larger than that of the homogenate. This is interpreted as a Pasteur effect associated with oxidations carried out by the particles.

When succinate is used as substrate, without addition of hexose diphosphate, the oxygen uptake of the particles is substantially higher than when pyruvate plus HDP is employed (Table III), being least with the tumor material, intermediate with brain, and highest with liver. The oxygen uptakes of the homogenates are also in the same order and in similar proportions. It is important to note that the endogenous lactate formation without added hexose diphosphate is apparently very low with all the tissues, as shown by the lactate values with succinate as substrate. In confirmation of earlier investigators, the aerobic phosphorylation by the tumor or brain homogenates with succinate as substrate is higher than that of the sum of the individual fractions (Table III).

2. *Action of dinitrocresol on phosphorylation of washed particles and phosphorylation of supernates.* The initial stimulus for these experiments was the observation that phosphorylation by tumor homogenates under aerobic conditions was resistant to or only slightly affected by concentrations of dinitrocresol (DNC) which completely suppressed aerobic phosphorylation of particles derived from liver and other normal tissues. In the experiments reported in Table IV the effects of various concentrations of dinitrocresol upon phosphorylation, lactate production, and oxygen uptake by washed particles of rat tumor, brain, and liver are compared with those exerted by the same concentrations of reagent upon carefully centrifuged, water-clear supernates derived from the same tissues. Since the purpose of this experiment was to determine the effect of DNC on particles freed as far as possible from supernate and on supernates completely freed of

particles and since it appeared desirable to use two different substrates (succinate, which might be expected to give optimum results for an oxidative phosphorylating system, and pyruvate plus HDP, which might be expected to give optimum results for an anaerobic, glycolytic, phosphorylating mechanism), no attempt was made in this experimental series to make simultaneous observations on the homogenates from which the particles and supernates were derived.

In confirmation of earlier work on particles from mammalian tissues(4,5) and from marine eggs(11), phosphorylation associated with oxidation of succinate by brain or liver particles was found to be completely suppressed by 10^{-5} M dinitrocresol. Aerobic phosphorus uptake by brain particles was markedly inhibited and by liver particles completely suppressed by 10^{-5} M DNC with hexose diphosphate and pyruvate as substrate. The lactate production in all these cases was low and the oxygen consumption relatively high. In the case of the Walker 256 rat tumor the particles showed no phosphorus uptake or lactate production with succinate. This was confirmed by a repeat experiment, but, as shown in Table III, tumor particles may give a moderate phosphorus uptake with succinate.

These differences may, as previously indicated, depend on differences in the state of the tumor and differences in the phosphate ester decomposing effect, which appears to be associated with tumor particles, and possibly also to the unavoidable inclusion of a certain amount of normal tissue. However, with pyruvate and HDP as substrate the tumor particles show a moderate uptake, which is only partially suppressed by DNC, and this phosphorus uptake appears to be associated with lactate formation substantially in excess of that observed in the corresponding experiments with brain and liver particles. The supernates with succinate show little or no phosphorus uptake or lactate production. However, the supernates of all 3 tissues show a very substantial phosphorus uptake when pyruvate plus HDP is employed as substrate. This phosphorus uptake is either unaffected or slightly increased by DNC, the highest phosphorus uptake in the case of tumor and brain supernates being associated with the highest concentration of DNC. The production of lactate is also substantial and virtually unaffected by DNC.

It would appear from these results that DNC at a concentration of 10^{-5} M completely blocks oxidative phosphorylation of brain and liver particles with succinate as substrate and also completely blocks the phosphorus uptake of liver particles with pyruvate plus HDP as substrate. However, when pyruvate plus HDP is used as substrate, 10^{-5} M DNC appears to reduce phosphorylation by brain particles to one-sixth and that by tumor particles to five-sixths of that observed without the addition of DNC. This observation lends support to the suggestion resulting from the observations on aerobic and anaerobic phosphorylation by tumor particles that a substantial amount of the water-soluble, anaerobic, glycolytic mechanism remains associated with the tumor particles, even after extensive washing by centrifugation, and contributes to the phosphorylating effects exerted by these particles.

In the experiments reported in Table V the effects of various concentrations of dinitrocresol upon phosphorylation, lactate production, and oxygen uptake by homogenates of rat tumor, brain, and liver are compared with those exerted on supernates obtained by centrifuging the homogenates in question. The DNC exerts a maximum effect on the liver homogenate, which at the highest concentration is reduced to 24%, an intermediate effect on the brain homogenate, which is reduced to 45%, and the minimum effect on the tumor homogenate, which is reduced to 90%, or 80% if a lower concentration of DNC is selected. The lactate production in micromoles, which appears to be unaffected by DNC, is in a similar order: 23% of the phosphate uptake in micromoles for liver homogenate, 42% for brain, and 88% for tumor homogenate, thus supporting the conclusion that the extent to which the phosphorylation

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11. Clowes, G. H. A., Keltch, A. K., Strittmatter, C. F., and Walters, C. P., *J. Gen. Physiol.*, 1950, v33, 555.

of a given homogenate survives the action of DNC affords an approximate index of the proportion of the anaerobic, glycolytic, phosphorylating mechanism functioning in the system in question. The oxygen uptake, which is in the reverse order, conforms with this concept. The phosphorylation of the supernates is found in all cases to be either unaffected or increased by the action of the DNC.

At this point reference should be made to an individual experiment in which the relation between the phosphorus uptake of a tumor homogenate and the supernate derived therefrom differed from those observed in the experiments reported above. A Walker tumor homogenate prepared in exactly the same manner as that employed in the experiment reported in Table V showed a phosphorus uptake of 13.4 with reduction under the influence of the concentrations of DNC employed in Table IV to 10.6, 10.6, 10.3, and 9.8, respectively. However, the supernate prepared from this homogenate in exactly the same manner as that employed in the experiments reported in Table V showed a phosphorus uptake of 17.1 and under the influence of the four concentrations of DNC values of 16.8, 16.1, 16.8, and 16.2, respectively. These values were associated with a very high lactate formation and low oxygen uptake. Obviously, in this case the phosphorus uptake of particles plus supernate would have exceeded that of homogenate, as with brain or liver.

In the concluding experiment reported in Table VI, observations were made on the phosphorus uptake and lactate formation of a tumor homogenate and a supernate derived therefrom under aerobic and anaerobic conditions similar to those reported in Table I, but in addition the effect exerted by two concentrations of DNC on the phosphorus uptake and lactate formation was determined. The phosphorus uptakes of the homogenate and supernate under anaerobic conditions and of the supernate under aerobic conditions were found, as in the experiment previously reported, to be the same within the range of experimental error and their average was 66 per cent of the phosphorus uptake of the homogenate under aerobic conditions. The effect of the dinitrocresol was to slightly reduce the phosphorus uptake of the homogenate under aerobic conditions and to increase the phosphorus uptake of the supernate under aerobic conditions and of the homogenate under anaerobic conditions, with the result that the phosphorus uptake in all 3 cases was the same within the range of experimental error. That of the supernate under anaerobic conditions was increased, but not to the same extent as the homogenate under anaerobic conditions.

In conclusion reference should be made to the observations of LePage and Schneider (10) on anaerobic phosphorylation of a homogenate of a Flexner-Jobling carcinoma and fractions derived therefrom in comparison with a normal liver homogenate and similar fractions. The tumor homogenate showed under these anaerobic conditions a high phosphorus uptake, while the liver homogenate gave only 6% of this amount and may therefore be considered to be negative. The tumor supernate appeared to account for about 60% and the nuclei for 33% of the phosphorus uptake of the homogenate under these anaerobic conditions. The mitochondria and microsomes showed a negative phosphorus uptake, which supports the conclusion that a negative phosphorus uptake of tumor particles may be due to the fact that under certain experimental conditions the decomposition of phosphate ester may exceed its synthesis.

LePage and Schneider's observations regarding the phosphorus uptake of tumor homogenates, nuclei, and supernates under anaerobic conditions give support to the suggestion that a part of the so-called aerobic phosphorylation induced by tumor particles may be attributable to the anaerobic glycolytic mechanism remaining associated with the tumor particles, even after the major part of this water-soluble, glycolytic mechanism has been removed by washing and centrifuging. This question will be made the subject of a future investigation.

Summary. 1. In liver and brain there are two distinct mechanisms for phosphorylation, which may be separated by centrifugation of the tissue homogenates: (1) particulate elements dependent on oxidation of succinate or other substrates of the tricarboxylic acid cycle and (2) water-soluble or at least water-borne systems glycolyzing hexose diphosphate. In the Walker 256 tumor the major part of the phosphorylating effect appears to be induced by the water-soluble, hexose diphosphate glycolyzing system, which functions equally well under aerobic and anaerobic conditions. 2. While the oxidative phosphorylation carried out by liver and brain particles with succinate or α-ketoglutarate is readily blocked by extremely dilute solutions of dinitrocresol, the water-borne, glycolytic, phosphorylating system which passes into the supernate on centrifuging the homogenate is not suppressed by dinitrocresol, even when used at concentrations from 100- to 500-fold those which completely suppress aerobic phosphorylation of washed liver particles. The phosphorus uptake of the tumor particles, which in any case is substantially less than that of liver and brain particles, is only partially suppressed under anaerobic conditions. Furthermore, a substantial part of the activity appears to be maintained at concentrations of DNC which completely block phosphorus uptake by liver particles. The phosphorus uptake of the tumor supernate is, like that of the liver and brain supernates, entirely unaffected by concentrations of DNC 100- to 500-fold those required to suppress the oxidative phosphorylation of liver particles. 3. The peculiar behavior of tumor particles under aerobic conditions and also when exposed to certain concentrations of DNC suggests the possibility that at least a part of the phosphorus uptake of tumor particles may be attributable to the anaerobic phosphorylating mechanism, which, even after extensive washing, remains attached to or associated with the tumor particles.

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